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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	6923-084	*Total Pages*	61
First Named Inventor or Application Identifier			
Chen et al.			
Express Mail Label No.	EM 061 023 892 US		

APPLICATION ELEMENTS
See MPEP chapter 600 concerning utility patent application contents.

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Box Patent Application
Washington, DC 20231

1. ☒ Fee Transmittal Form
   Submit an original, and a duplicate for fee processing)
2. ☒ Specification [*Total Pages* 48]
   (preferred arrangement set forth below)
   - -Descriptive title of the Invention
   - -Cross Reference to Related Applications
   - -Statement Regarding Fed sponsored R&D
   - -Reference to Microfiche Appendix
   - -Background of the Invention
   - -Brief Summary of the Invention
   - -Brief Description of the Drawings *(if filed)*
   - -Detailed Description of the Invention (including drawings, *if filed*)
   - -Claim(s)
   - -Abstract of the Disclosure
3. ☒ Drawing(s) *(35 USC 113)* [*Total Sheets* 9]
4. ☒ Oath or Declaration [*Total Sheets* 2]
   a. ☐ Newly executed (original or copy)
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   (for continuation/divisional with Box 17 completed)
   [Note Box 5 below]
   i. ☐ DELETION OF INVENTORS(S)
   Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33 (b).
5. ☐ Incorporation By Reference *(useable if Box 4b is checked)*
   The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
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8. ☐ Assignment Papers (cover sheet & document(s))
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ATTORNEY DOCKET NO. 6923-084-999 Date: January 14, 2000

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Sir:

The following utility patent application is enclosed for filing:

Applicant(s): Shu-Hsia Chen and Savio L.C. Woo Executed on:

Title of Invention: COMBINATION THERAPY OF CANCER BY THE ACTIVATION OF CO-STIMULATORY MOLECULES EXPRESSED BY IMMUNE CELLS AND CYTOKINES

PATENT APPLICATION FEE VALUE

TYPE	NO. FILED	LESS	EXTRA	EXTRA RATE	FEE
Total Claims	54	-20	34	$18.00 each	$ 612.00
Independent	3	-3	0	$78.00 each	$ 0.00
			Minimum Fee		$ 690.00
			Multiple Dependency Fee If Applicable ($260.00)		$ 260.00
			Total		$ 1,562.00
			50% Reduction for Independent Inventor, Nonprofit Organization or Small Business Concern (a verified statement as to the applicant's status is attached)		- $ 0.00
				Total Filing Fee	$ 1,562.00

☒ Priority of application no. 60/115,992 filed on January 15, 1999 is claimed under 35 U.S.C. § 119.
☐ The certified copy of the priority application has been filed in application no. filed
☐ Amend the specification by inserting before the first line the following sentence: This is a continuation-in-part of application no. filed .

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Respectfully submitted, by Jacqueline Bonn
Reg No 43,492
Laura A. Coruzzi 30,742
Laura A. Coruzzi (Reg. No.)
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COMBINATION THERAPY OF CANCER BY THE ACTIVATION OF CO-STIMULATORY MOLECULES EXPRESSED BY IMMUNE CELLS AND CYTOKINES

1. FIELD OF THE INVENTION

The present invention the present invention relates to methods and compositions for the treatment, prevention or inhibition of diseases and disorders, including cancer, inflammatory diseases or disorders, and infectious diseases, comprising compounds which augment activated immune cells, *i.e.*, T-cells and natural killer ("NK") cells. In particular, the present invention relates to methods and compositions for the treatment, prevention or inhibition of diseases and disorders, including cancer, inflammatory diseases or disorders, and infectious diseases, comprising the administration of a compound that activates one or more cytokine receptors and a compound that activates of one or more co-stimulatory molecules expressed by activated immune cells, *i.e.*, T-cells.

2. BACKGROUND OF THE INVENTION

A neoplasm, or tumor, is a neoplastic mass resulting from abnormal uncontrolled cell growth, which can be benign or malignant. Benign tumors generally remain localized. Malignant tumors are collectively termed cancers. The term "malignant" generally means that the tumor can invade and destroy neighboring body structures and spread to distant sites and cause death (for review, see Robins and Angell, 1976, *Basic Pathology*, 2d Ed., W.B. Saunders Co., Philadelphia, pp. 68-122). A tumor is said to have metastatized when it has spread from one organ or tissue to another.

Cancer is the second leading cause of deaths in the United States. Carcinoma of the colon and rectum is second only to lung cancer as a major cause of cancer deaths. Prognosis for patients with metastatic disease in the liver and other organs is poor, and with current treatment, the mean survival time is only 3.7 years (Dreben, J.A. and Niederhuber, J.E., 1993, Colon Cancer In: Current Therapy in Oncology. Niederhuber, J.A. ex., B.C. Decker, St. Louis, 426-431; Lebovic, G.S., and Niederhuber, J.E., 1993, Colorectal cancer metastatic to the liver: Hepatic arterial infusion. In: Current Therapy in Oncology. Niederhuber, J.E., ed. B.C. Decker, St. Louis. 389-395; Fortner J.G., 1993, Colorectal cancer metastatic to the liver: Surgical Resection. In: Current Therapy in Oncology. Niederhuber, J.E., ed. B.C. Decker, St. Louis; Kemeny, N. and Selter, K., 1993, Metastatic Colorectal Cancer: Chemotherapy. In: Current Therapy in Oncology. Niederhuber, J.E., ed. B.C. Decker, St. Louis. pp. 447-456). Therefore, a need exists for the development of alternative treatments for metastatic carcinoma than currently available.

One approach to the treatment of metastatic carcinoma is *ex vivo* gene therapy. In the *ex vivo* gene therapy or "cancer vaccine" approach, cancer cells are isolated from patients, transduced with various gene vectors and expanded *in vitro*. After irradiation, the cells are transplanted autologously to enhance the patient's immune response against the tumor. This strategy is not only laborious but the treatment is also individualized as cancer cells need to be cultured and expanded from each patient for therapeutic purposes. A more attractive strategy is to deliver the cytokine genes *in vivo*.

Cancer immunotherapy is a potent approach to combat metastatic diseases by stimulating a systemic anti-tumor response against disseminated tumor cells in the host. One reagent that has been shown to possess some anti-tumor activity when administered at the site of some murine tumors is B7-1 (Wu et al., 1995, *J. Exp. Med.* 182: 1415-1421; Chen et al., 1994, *J. Exp. Med.* 179: 523-532). B7-1 is a ligand expressed on the surface of antigen-presenting cells (APCs) that binds to the CD28 receptor expressed on the surface of resting T-cells. The ability of B7-1 expression to induce an anti-tumor response is dependent on the type of tumor. Thus, B7-1 mediated immunotherapy is limited in its effectiveness in treatment of cancer.

One of the most promising reagents in cancer treatment to date is interleukin-12 (IL-12) due to its multiple regulatory effects. IL-12 is produced by antigen presenting cells (APC) such as macrophages, dendritic cells and B cells following appropriate stimulation. It plays an important role in orchestrating the host immune response by inducing interferon (IFN)-γ expression, promoting Thl cell differentiation, and enhancing T-cell, natural killer (NK) cell, lymphokine-activated killer (LAK), and macrophage mediated cytolytic activity (Banks et al., 1995, *Br. J. Cancer* 71: 655-659; Brunda, M.J., 1994, Interleukin-12. *J. Leukocyte Biology* 55: 280-288; Tsung et al., 1997, *J. Immunology* 158: 3359-3365; Scott, P., 1993, *Science* 260: 496-497; Nishmura et al., S., 1995, *Immunology Letter* 48: 167-174; Takeda et al., 1996, *J. Immunology* 156: 3366-3373; Cesano et al., 1993, *J. Immunology* 154: 2943-2957). In particular, IFN-γ induced IL-12 has been shown to enhance APC functions that are critical for IL-12 mediated therapy.

Caruso et al. demonstrated that intratumoral administration of a recombinant adenoviral vector expressing the murine IL-12 (Adv.mIL-12) results in high level expression of IL-12 at the tumor site and induces a strong anti-tumor immune response in a well established orthotopic murine colon carcinoma (MCA26) liver metastases model in syngeneic Balb/c mice (Caruso, M., Pham-Nguyen, K., Kwong, Y.L., Xu, B., Kosai, K.I., Finegold, M., Woo, S.L.C., and Chen, S.H., 1996, *Proc. Natl. Acad. Sci.* 93: 11302-11306). However, at the high doses of IL-12 gene expression needed to induce the long-term regression of established tumor, vector mediated IL-2 gene expression is toxic in animals (Putzer et al., 1997, *Proc. Natl. Acad. Sci., USA* 94: 10889-10894). Thus, vector mediated IL-12 gene application within a tumor is not effective in achieving tumor rejection.

Recently, Putzer et al. demonstrated that intratumoral administration of murine IL-12 and B7-1, a ligand for the co-stimulatory molecule CD28 which is expressed on resting T-cells, induces the regression of established tumors in a transgenic murine model of metastatic breast cancer and results in protective immunity against a second challenge with tumor cells (Putzer et al., 1997, *Proc. Natl. Acad. Sci., USA* 94: 10889-10894).

2.1 Co-stimulatory Molecules

Co-stimulatory molecules such as 4-1BB, signaling lymphocyte activation molecule (SLAM), and OX-40 are expressed only or predominantly on activated T-cells. These co-stimulatory molecules have been suggested to act at different stages of T-cell activation or differentiation than CD28, or to promote the development of different effector functions than CD28 (Vinay et al., 1998, *Seminars Immunology* 10: 481-489; Aversa et al., 1997, *J. Immunology* 158: 4036-4044; Weinberg et al., 1998, *Seminars Immunology* 10: 471-480).

SLAM (or CDw150) is a member of the CD2 subfamily of the immunoglobulin superfamily and is expressed on the surface of activated T- and B-cells. SLAM upregulates IFN-γ and seems to act only on memory cells (Aversa et al., 1997, *J. Immunology* 158: 4036-4044).

OX-40 (or CD134) expression is a member of the tumor necrosis factor receptor (TNFR) superfamily that binds to OX-40 ligand (OX-40L) expressed on antigen presenting cells, such as activated B-cells and dendritic cells. OX-40 expression is limited to activated $CD4^+$ T-cells. Co-stimulation of T-cells through OX-40 enhances T-cell proliferation and cytokine production. OX-40 has been suggested to play a role in sustaining proliferation of Th1 or Th2 effector cells and promoting the development of a Th2 response (Weinberg et al., 1998, *Seminars Immunology* 10: 471-480).

4-1BB glycoprotein is a member of the TNFR superfamily that binds to a high affinity ligand (4-1BB ligand) expressed on antigen presenting cells (APCs), such as dendritic cells, macrophages and activated B-cells (Vinay et al., 1998, *Seminars Immunology* 10: 481-489). Expression of 4-1BB s restricted to primed CD4+ and CD8+ T-cells (Goodwin, R.G., et al., 1993, *Eur. J. Immunol.* 23: 2631-2641; Pollok, K.E., et al., 1993, *J. Immunol.* 150: 771-781) after antigen or mitogen induction. Its interaction with 4-1BB ligand provides a strong signal for expansion of TCR ligated T-cells. It has been shown that systematic administration of an agonistic monoclonal antibody causes tumor reduction in s.c. tumor bearing animals, and both CD4+ and CD8+ T-cells are involved in the anti-tumor response (Melero et al., 1997, *Nature Med.* 3: 682-685; Melero et al., 1998, *Eur. J. Immunol.* 28: 1116-1121). However, anti-4-1BB antibody treatment is not adequate to sustain long term immunity.

3. SUMMARY OF THE INVENTION

The present invention encompasses therapeutic methods and compositions for the treatment, prevention or inhibition of diseases and disorders, including cancer, inflammatory diseases or disorders, infectious diseases (*e.g.*, microbial and viral infections) and diseases of the immune system, comprising compounds which augment activated immune cells, *i.e.*, T-cells and natural killer ("NK") cells. In particular, the present invention relates to methods and compositions for the treatment, prevention or inhibition of diseases and disorders, including cancer, inflammatory diseases or disorders, and infectious diseases, comprising the administration of a compound (*i.e.*, a cytokine or antibody) that activates one or more cytokine receptors and a compound (*i.e.*, a ligand or antibody) that activates of one or more co-stimulatory molecules expressed by activated immune cells, *i.e.*, activated T-cells.

Therapeutic and pharmaceutical compositions of the present invention comprise: (i) one or more cytokines in combination with one or more ligands for co-stimulatory molecules expressed on activated immune cells; (ii) one or more cytokines in combination with antibodies which bind to and activate co-stimulatory molecules expressed on activated immune cells; (iii) antibodies which interact and activate cytokine receptors in combination with one or more ligands for co-stimulatory molecules expressed on activated immune cells; and (iv) antibodies which bind to and activate co-stimulatory molecules expressed on activated immune cells in combination with antibodies which bind to and activate cytokine receptors. The polypeptides can be supplied by direct administration or indirectly as "pro-drugs" using somatic cell gene therapy. The pharmaceutical compositions of the present invention are in suitable formulation to be administered to animals, preferably mammals such as companion animals (*e.g.*, dogs, cats, and horses) and livestock (*e.g.*, cows and pigs), and most preferably humans. The pharmaceutical compositions are administered in an amount effective for the treatment, prevention or inhibition of a disease or disorder such as cancer and an infectious disease, or an amount effective for inducing an anti-tumor response (*e.g.*, the inhibition of the hyperproliferation of a tumor), or an amount effective for reducing viral load or bacterial numbers, or an amount effective for augmenting activated immune cells, or an amount effective such that the immune response in a subject (an animal) is augmented.

3.1. DEFINITIONS

The term "activated immune cells" as used herein refers to activated lymphoid cells (*e.g.*, T-cells, natural killer (NK) cells, B-cells), activated myeloid cells (*e.g.*, macrophages, monocytes, eosinophils, neutrophils, basophils, mast cells, granulocytes and platelets), activated dendritic cells, and activated antigen presenting cells. Immune cells can determined to be activated based on the expression of specific activation markers (antigens) or the production of specific cytokines. The expression of activation markers and cytokines

can be determined by a variety of methods known to those of skill in the art, including immunofluorescence, and fluorescence activated cell- sorter (FACS) analysis, western blot analysis, northern blot analysis, RT-PCR.

The term "activated T-cells" as used herein refers T-cells expressing antigens indicative of T-cell activation (T-cell activation markers). Examples of T-cell activation markers include, but are not limited to, CD25, CD26, CD30, CD38, CD69, CD70, CD71, ICOS, OX-40 and 4-1BB. The expression of activation markers can be measured by techniques known to those of skill in the art, including, for example, western blot analysis, northern blot analysis, RT-PCR, immunofluorescence assays, and fluorescence activated cell sorter (FACS) analysis.

The term "resting T-cells" as used herein refers to T-cells which do not express T-cell activation markers. Resting T-cells include, but are not limited to, T-cells which are $CD25^-$, $CD69^-$, $ICOS^-$, $SLAM^-$, and $4\text{-}1BB^-$. The expression of these markers can be measured by techniques known to those of skill in the art, including, for example, western blot analysis, northern blot analysis, RT-PCR, immunofluorescence assays, and fluorescence activated cell sorter (FACS) analysis.

The term "augment" as used herein refers to an increase in the biological activity (*e.g.*, the proliferation, differentiation, priming, effector function, production of cytokines or expression of antigens) of activated immune cells. In particular, a compound that augments an activated T-cell activates an activated T-cell 1-5 fold, 5-10 fold, 10-20 fold or more than 20 fold as compared to the ability of the compound to activate a resting T-cell as determined by assays known to those of skilled in the art, in particular those assays described in Section 5.5 which measure the proliferation and the expression of cytokines and antigens.

The term "selectively activates activated T-cells" as used herein refers to activating activated T-cells to a substantially greater degree when compared to activating resting T-cells as determined by assays known to those of skill in the art, in particular those assays described in Section 5.5. In particular, a compound that selectively activates activated T-cells refers to a compound that activates an activated T-cell 1-5 fold, 5-10 fold, 10-20 fold or more than 20 fold as compared to the ability of the compound to activate a resting T-cell as determined by assays known to those skilled in the art, in particular those assays described in Section 5.5 which measure the proliferation and the expression of cytokines and antigens.

The term "cytokine" as used herein relates to native or recombinant secreted low molecular weight polypeptides, peptides, fragments, derivatives or analogs thereof that modulate the activity (*i.e.*, proliferation and differentiation) of immune cells. Examples of cytokines include, but are not limited to, IFN-α, IFN-β, IFN-γ, TNF-α, interleukin-1β (IL-1β), IL-2, interleukin-3 (IL-3), IL-4, IL-5, IL-10, IL-12, interleukin-15 (IL-15), interleukin-18 (IL-18), granulocyte colony-stimulating factor (G-CSF), GM-CSF and chemokines such

as macrophage inflammatory protein-1 (MIP-1), gamma interferon inducible protein (IP-10) and monkine induced by IFN-γ (MIG).

In accordance with the present invention, pharmaceutical compositions comprising ligands for co-stimulatory molecules encompass pharmaceutical compositions comprising native or recombinant polypeptides, peptides, fragments, derivatives or analogs thereof that interact with and activate co-stimulatory molecules expressed by activated T-cells. Examples of ligands for co-stimulatory molecules expressed on activated T-cells, referred to herein as ligand or ligands, include, but are not limited to, 4-1BBL, SLAM, CD7O ligand (CD70L) and OX-40L, with the proviso that the ligand is not B7-1. In accordance with the present invention, co-stimulatory molecules expressed by activated T-cells which are encompassed by the present invention include, but are not limited to, SLAM, OX40, 4-1BB, inducible co-stimulator (ICOS), B7RP-1 and CD27.

4. DESCRIPTION OF THE FIGURES

Figure 1. Survival of tumor bearing animals after ADV.mIL-12 and anti-41BB treatment. Animals were intrahepatically implanted with $7x10^4$ MCA26 tumor cells for 7 days. The animals with hepatic tumor sizes of $5x5mm^2$ were divided into several groups. the groups were injected intratumorally with various doses of Adv.mIL-12 ($3.2x10^8$ pfu, n=8; $1.6x10^8$pfu, n=8; 0.8x108 pfu; n=12; $0.4x10^8$pfu, n=5; $0.2x10^8$pfu,, n=5; and $0.1x10^8$pfu, n=5) or a control vector, DL312 ($3.2x10^8$pfu, n=12) in combination with anti-4-1BB or a control antibody. The antibodies were injected intraperitoneally at days 8 and 11 at a does of 50μg/mouse. Survival difference between combination IL-12 ($3.6x10^8$pfu)+anti-4-1BB treated animals was statistically significant from either DL312+anti-4-1BB (n=12) or (ADV.mIL-12+control Ig ($3.2x10^8$pfu, n=12) treated animals by Logrank survival analysis ($p<0.0001$). The results reported here were pooled from two consecutive sets of experiments.

Figure 2. Long-term survival study of BALB/c mice bearing JC breast carcinoma liver metastases treated with ADV/IL-12 + anti-4-1BB antibody. Animals bearing tumors 5x5 mm in diameter were attributed to four groups (n=15-25 animals/group): 1) (◆) ADV/IL-12 ($1x10^8$ pfu/animal) + anti-4-1BB (2x50 μg i.p.); 2) (▲) ADV/IL-12 ($3.6x10^8$ pfu/animal); 3) (■) ADV/D1312 + anti-4-1BB; 4) (X) ADV/DL312 + control Ig. 87% of the combination IL-12 plus anti-4-1BB treated animals showed long-term survival while 60% of the anti-4-1BB treated animals did so (P=0.02, logrank test). In the IL-12 group, 22% of the mice survived while 100% of the control animals died within 60 to 70 days after tumor inoculation.

Figure 3. Combination adenoviral mediated gene therapy of IL-12 and 4-1BB ligand. Animals with hepatic tumor at size 5x5 mm^2 were divided into four groups, and each group (n=5-7) were injected intratumorally with various doses of Adv.m4-1BB ligand

($1x10^9$ and $0.5x10^6$ pfu) in combination with Adv.mIL-12 ($2x10^8$ pfu) or control vector, DL312 ($2x10^8$ pfu). The survival difference between the combination of IL-12 and 4-1BB ligand treated animals was statistically significant than either Adv.m4-1BB ligand and DL312 or Adv.mIL-12 and DL312 treated animals by Logrank survival analysis ($p<0.042$).

Figure 4. Long-term survival study of BALB/c mice bearing JC breast carcinoma liver metastases treated with ADV/IL-12 + ADV/4-1BBL. Animals bearing tumors 5x5 mm in diameter were attributed to four groups: 1) (◆) ADV/IL-12 ($1x10^8$ pfu/animal) + ADV/4-1BBL ($1x10^9$ pfu/animal); 2) (■) ADV/IL-12 ($1x10^8$ pfu/animal) + ADV/DL312 ($1x10^9$ pfu/animal); 3) (▲) ADV/D1312 + anti-4-1BB ($1x10^9$ pfu/animal); 4) (X) ADV/DL312 ($1.1x10^9$ pfu/animal). 78% of combination IL-12 + 4-1BBL treated animals showed long-term survival. Compared to IL-12 (22% survival) or 4-1BBL (13% survival) alone, the difference is statistically significant with P values of 0.016 and 0.004, respectively (logrank test).

Figure 5. Subcutaneous challenge of long-term surviving animals after JC liver metastases treatment. Surviving (>120 days after tumor cell inoculation) animals after treatment with ADV/IL-12 or anti-4-1BB antibody alone, or combination ADV/IL-12 + anti-4-1BBL received a s.c. injection of JC parental cells or MCA26 cells. Formation of tumor was observed over a 4-week period. Naive animals were also injected to assess the normal growth pattern of the 2 tumors. Various percentages of animals in the long-term surviving groups did not form any tumor. However, only the results of the ADV/IL-12 + ADV/4-1BBL group reached statistical significance compared to naive controls (P=0.007, Fischer's exact test). Conversely, the rate of JC tumor growth was dramatically reduced among all surviving animals.

Figure 6. Effect of hepatic tumor combination treatment on macroscopic lung metastases of colon carcinoma. An animal model with both liver tumor and pre-established multiple macroscopic tumor nodules in the lung was subjected to test the systemic anti-tumor effect. Control animals receiving no treatment developed multiple lesions in the lung, and all of them died within 32 days. 100% of the liver and lung tumor bearing animals receiving the combination treatment ($0.4x\ 10^8$pfu Adv. mIL-12 +anti 4-1BB) in the liver tumor (n=6) survived well after 70 days. The results indicate distant protection against pre-existing macroscopic lung metastases by hepatic tumor combination treatment ($p<0.0011$) by Logrank test.

Figure 7. (A) Evaluation of cellular immune response in Adv.mIL-12 ($0.4x10^8$pfu) and anti-4-1BB treated animals. MNC were isolated from animals at days 0, 1, 2, 4,7 and 14 (five mice per time point per group) after treatments and the cells were assayed for direct cytolytic killing against ^{51}Cr labeled parental MCA26 tumor cells. Direct tumor cell killing activity can be seen at days 2 and 4 in ADV.mIL-12+anti-4-1BB treated animals, and only low activity was present in anti-4-1-BB alone or ADV.mIL-12 alone treated animals. The

standard deviation of the triplicate wells is less than 7% (B) Identification of effector cell types by in vitro depletion of effector cells. MNC isolated from combination treated animals at day 2 were divided and depleted of NK, CD4+T or pan-T cells, using purified Dx5 antibody, GK1.5 and Thy1.2 hybridoma supernatant, respectively, that were conjugated with complement The control group was treated with complement alone. Less than 1% of T cells remained after depletion as confirmed by FACS staining, and less than 5% of NK activity remained as confirmed by YAC-1 killing. The standard deviation of the triplicate wells is usually less than 7%.

Figure 8. Effect of leukocyte depletion on tumor rechallenge in long-term surviving animals. Long-term surviving animals were depleted of NK (n=8) cells at optimal conditions and with appropriate controls, including non-tumor bearing naive (n=8) and control IG (n=7), prior to being challenged by subcutaneous injection of parental MCA26 tumor cells ($7x10^8$). Over a four-week observation period, 100% of the non-tumor bearing native animals formed subcutaneous tumor, and only 14.2% of control Ig injected mice formed tumor. In the NK deleted group, 87.5% of the animals formed MCA26 tumor, and 100% of the CD8+T cell depleted animals formed tumor. (*) indicates statistical significance when compared to control Ig treated group by Fisher Exact test.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention is based, in part, on Applicants' discovery that 4-1BB ligand or anti-4-1BB antibody and IL-12 act synergistically and result in a dramatic improvement in the therapeutic outcome of hepatic colon carcinoma and lung metastases in mice compared to either 4-1BB ligand alone, anti-4-1BB antibody alone or IL-12 alone. The present invention encompasses therapeutic methods and pharmaceutical compositions for the treatment, prevention or inhibition of diseases and disorders, including cancer, inflammatory diseases or disorders, infectious diseases and diseases of the immune system, comprising compounds which augment or enhance activated immune cells, *i.e.*, T-cells and natural killer ("NK") cells. In particular, the present invention relates to methods and pharmaceutical compositions for the treatment, prevention or inhibition of diseases and disorders, including cancer, inflammatory diseases or disorders, and infectious diseases, comprising the administration of a compound (*i.e.*, a cytokine or antibody) that activates one or more cytokine receptors and a compound (*i.e.*, a ligand or antibody) that activates of one or more co-stimulatory molecules expressed by activated immune cells, *i.e.*, activated T-cells. In yet another embodiment, the present invention relates to methods and pharmaceutical compositions comprising a compound that activates one or more cytokine receptors and a compound that selectively activates activated T-cells (*e.g.*, T-cells expressing ICOS, SLAM, CD25, CD30 and OX-40). In a preferred embodiment, the methods and pharmaceutical

compositions of the present invention are for the treatment, prevention or inhibition of cancer.

Therapeutic and pharmaceutical compositions of the present invention comprise:

(i) polynucleotide sequences encoding one or more cytokines in combination with polynucleotide sequences encoding one or more ligands for co-stimulatory molecules expressed on activated T-cells;

(ii) polynucleotide sequences encoding one or more cytokines in combination with polynucleotide sequences encoding one or more antibodies which bind to co-stimulatory molecules expressed on activated T-cells and induce the co-stimulatory molecule's signaling pathway;

(iii) polynucleotide sequences encoding one or more cytokines in combination with polypeptides coding for one or more ligands which interact with co-stimulatory molecules expressed on activated T-cells;

(iv) polynucleotide sequences encoding one or more cytokines in combination with one or more antibodies which bind to co-stimulatory molecules expressed on activated T-cells and induce the co-stimulatory molecule's signaling pathway;

(v) polypeptide sequences coding one or more cytokines in combination with polynucleotide sequences encoding one or more ligands for co-stimulatory molecules expressed on activated T-cells;

(vi) polypeptide sequences coding one or more cytokines in combination with polynucleotide sequences encoding antibodies which bind to one or more co-stimulatory molecules expressed on activated T-cells and induce the co-stimulatory molecule's signaling pathway;

(vii) polypeptide sequences coding one or more cytokine in combination with one or more polypeptides coding for one or more ligand s which interact with co-stimulatory molecules expressed on activated T-cells;

(viii) polypeptide sequences coding one or more cytokines in combination with antibodies which bind to one or more co-stimulatory molecules expressed on activated T-cells and induce the co-stimulatory molecule's signaling pathway;

(ix) polynucleotide sequences encoding antibodies which activate one or more cytokine receptors in combination with polynucleotide sequences encoding one or more ligands for co-stimulatory molecules expressed on activated T-cells;

(x) polynucleotide sequences encoding antibodies which activate one or

more cytokine receptors in combination with polynucleotide sequences encoding antibodies which bind to one or more co-stimulatory molecules expressed on activated T-cells and induce the co-stimulatory molecule's signaling pathway;

(xi) polynucleotide sequences encoding antibodies which activate one or more cytokine receptors in combination with polypeptides coding for one or more ligands which interact with co-stimulatory molecules expressed on activated T-cells;

(xii) polynucleotide sequences encoding antibodies which activate one or more cytokine receptors in combination with antibodies which bind to one or more co-stimulatory molecules expressed on activated T-cells and induce the co-stimulatory molecule's signaling pathway;

(xiii) antibodies which activate one or more cytokine receptors in combination with polynucleotide sequences encoding one or more ligands for co-stimulatory molecules expressed on activated T-cells;

(xiiii) antibodies which activate one or more cytokine receptors in combination with polynucleotide sequences encoding antibodies which bind to one or more co-stimulatory molecules expressed on activated T-cells and induce the co-stimulatory molecule's signaling pathway;

(xiv) antibodies which activate one or more cytokine receptors in combination with polypeptides coding for one or more ligands which interact with co-stimulatory molecules expressed on activated T-cells; and

(xvi) antibodies which activate one or more cytokine receptors in antibodies which bind to one or more co-stimulatory molecules expressed on activated T-cells andinduce the co-stimulatory molecule's signaling pathway.

The pharmaceutical compositions of the present invention comprising cytokines include native or recombinant polypeptides, peptides, fragments, derivatives or analogs thereof of such cytokines as IFN-α, IFN-β, IFN-γ, TNF-α, interleukin-1β (IL-1β), IL-2, interleukin-3 (IL-3), IL-4, IL-5, IL-10, IL-12, interleukin-15 (IL-15), interleukin-18 (IL-18), granulocyte colony-stimulating factor (G-CSF), GM-CSF and chemokines. The pharmaceutical compositions comprising ligands for co-stimulatory molecules encompass pharmaceutical compositions comprising native or recombinant polypeptides, peptides, fragments, derivatives or analogs thereof interact with and activate co-stimulatory molecules expressed by activated T-cells. Examples of ligands for co-stimulatory molecules expressed on activated T-cells, referred to herein as ligand or ligands, include, but are not limited to, 4-1BBL, SLAM, CD7O ligand (CD70L) and OX-40L, with the proviso that the ligand is not B7-1. In accordance with the present invention, co-stimulatory molecules expressed by

activated T-cells which are encompassed by the present invention include, but are not limited to, SLAM, OX40, 4-1BB, ICOS, B7RP-1, and CD27.

The pharmaceutical compositions of the present invention are in suitable formulation to be administered to animals, preferably mammals such as companion animals (*e.g.*, dogs, cats, and horses) and livestock (*e.g.*, cows and pigs), and most preferably humans. The pharmaceutical compositions of the invention are administered to a subject (an animal) in an amount effective for the treatment, prevention or inhibition of a disease or disorder (*i.e.*, cancer and infectious diseases), or an amount effective for inducing an anti-tumor response (*e.g.*, the inhibition of the hyperproliferation of a tumor), or an amount effective for augmenting activated immune cells, or an amount such that the immune response of the subject (animal) is augmented.

In one embodiment, therapeutic methods and compositions for the treatment, prevention or inhibition of diseases or disorders, including infectious diseases and cancer, comprise a compound that activates one or more cytokine receptors and a compound that activates 4-1BB (*e.g.*, 4-1BB ligand and anti-4-1BB antibody). In accordance with this embodiment, a compound that activates one or more cytokine receptors can comprise one or more antibodies, one or more ligands, or the combination thereof. In another embodiment, therapeutic methods and compositions for the treatment, prevention or inhibition of diseases or disorders, including infectious diseases and cancer, comprise a compound that activates the IL-12 receptor (*e.g.*, IL-12 or anti-IL-12 antibodies) and a compound that activates one or more co-stimulatory molecules expressed on activated immune cells (*e.g.*, SLAM, OX40, 4-1BB, ICOS, B7RP-1, and CD27). In accordance with this embodiment, a compound that activates one or more co-stimulatory molecules can comprise one or more antibodies, one or more ligands, or the combination thereof. In yet another embodiment, therapeutic methods and compositions for the treatment, prevention or inhibition of diseases or disorders, including infectious diseases and cancer, comprise a compound that activates the IL-12 receptor (*e.g.*, IL-12 or anti-IL-12 antibodies) and one or more additional cytokines (*e.g.*, IFN-α, IFN-β, IFN-γ, TNF-α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-10, IL-12, IL-15, IL-18), and a compound that activates one or more co-stimulatory molecules expressed on activated immune cells (*e.g.*, SLAM, OX40, 4-1BB, ICOS, B7RP-1, and CD27). In a specific embodiment, the additional cytokines are IL-15 and IL-18.

In another embodiment, therapeutic methods and compositions for the treatment, prevention or inhibition of diseases or disorders, including infectious diseases and cancer, comprise a compound that activates the IL-15 receptor (IL-15R; *e.g.*, IL-15 or anti-IL-15 antibodies) and a compound that activates one or more co-stimulatory molecules expressed on activated immune cells (*e.g.*, SLAM, OX40, 4-1BB, ICOS, B7RP-1, and CD27). In another embodiment, therapeutic methods and compositions for the treatment, prevention or inhibition of diseases or disorders, including infectious diseases and cancer, comprise a

compound that activates the IL-18 receptor (IL-18R; *e.g.*, IL-18 or anti-IL-18 antibodies) and a compound that activates one or more co-stimulatory molecules expressed on activated immune cells (*e.g.*, SLAM, OX40, 4-1BB, ICOS, B7RP-1, and CD27). In another embodiment, therapeutic methods and compositions for the treatment, prevention or inhibition of diseases or disorders, including infectious diseases and cancer, comprise a compound that activates the IL-15 receptor and IL-18 receptor, and a compound that activates one or more co-stimulatory molecules expressed on activated immune cells (*e.g.*, SLAM, OX40, 4-1BB, ICOS, B7RP-1, and CD27). In yet another embodiment, therapeutic methods and compositions for the treatment, prevention or inhibition of diseases or disorders, including infectious diseases and cancer, comprise a compound that activates the IL-12 receptor, IL-15 receptor and IL-18 receptor, and a compound that activates one or more co-stimulatory molecules expressed on activated immune cells (*e.g.*, SLAM, OX40, 4-1BB, ICOS, B7RP-1, and CD27).

In another embodiment, therapeutic methods and compositions for the treatment, prevention or inhibition of diseases or disorders, including infectious diseases and cancer, comprise 4-1BB or anti-4-1BB antibody and IL-12. In accordance with this embodiment, an animal, preferably mammal and most preferably a human is administered a therapeutic or pharmaceutical composition comprising: (i) native or recombinant IL-12 polypeptides, peptides, fragments, derivatives or analogs thereof in combination with native or recombinant 4-1BBL polypeptides, peptides, fragments, derivatives or analogs thereof; (ii) native or recombinant IL-12 polypeptides, peptides, fragments, derivatives or analogs thereof in combination with anti-4-1BB antibodies, (iii) anti-IL-2 receptor (IL-12R) antibodies in combination with native or recombinant 4-1BBL polypeptides, peptides, fragments, derivatives or analogs thereof; and (iv) anti-IL-12R antibodies in combination with anti-4-1BB antibodies. The proteins can be supplied by direct administration or indirectly as "pro-drugs" using somatic cell gene therapy.

5.1 EXPRESSION OF DNA

The nucleotide sequence encoding a cytokine (*i.e.*, IFN-α, IFN-β, IFN-γ, TNF-α, interleukin-1β (IL-1β), IL-2, interleukin-3 (IL-3), IL-4, IL-5, IL-10, IL-12, interleukin-15 (IL-15), interleukin-18 (IL-18), granulocyte colony-stimulating factor (G-CSF), GM-CSF and chemokines) or a functionally active analogs or fragments or other derivatives thereof can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The nucleotide sequence encoding a ligand (*i.e.*, 4-1BBL, SLAM, CD7O ligand (CD70L) and OX-40L) for a co-stimulatory molecule expressed on activated T-cells or functionally active analogs or fragments or other derivatives thereof can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the

transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translational signals can also be supplied by the native cytokine or native ligand for a co-stimulatory molecule genes or its flanking regions. A variety of host-vector systems may be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used. In specific embodiments, the human IL-12 and human 4-1BB ligand genes are expressed, or sequences encoding functionally active portions of the human IL-12 and human 4-1BB ligand are expressed. In yet another embodiment, a fragment of target comprising a domain of the IL-12 and/or 4-1BB ligand are expressed.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional and translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). Expression of the nucleic acid sequence encoding a cytokine or ligand for a co-stimulatory molecule or peptide fragments thereof may be regulated by a second nucleic acid sequence so that the cytokine or ligand or peptide fragments thereof are expressed in a host transformed with the recombinant DNA molecule. For example, expression of IL-12 or 4-1BB ligand may be controlled by any promoter or enhancer element known in the art. Promoters which may be used to control cytokine and/or ligand gene expression include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, 1981, *Nature* 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, *Cell* 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, *Proc. Natl. Acad. Sci. USA* 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, *Nature* 296:39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff et al., 1978, *Proc. Natl. Acad. Sci. USA* 75:3727-3731), or the *tac* promoter (DeBoer et al., 1983, *Proc. Natl. Acad. Sci. USA* 80:21-25); see also "Useful proteins from recombinant bacteria" in *Scientific American*, 1980, 242:74-94; plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., *Nature* 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner et al., 1981, *Nucl. Acids Res.* 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, *Nature* 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase)

promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, *Cell* 38:639-646; Ornitz et al., 1986, *Cold Spring Harbor Symp. Quant. Biol.* 50:399-409; MacDonald, 1987, *Hepatology* 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, *Nature* 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, *Cell* 38:647-658; Adames et al., 1985, *Nature* 318:533-538; Alexander et al., 1987, *Mol. Cell. Biol.* 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, *Cell* 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, *Genes and Devel.* 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, *Mol. Cell. Biol.* 5:1639-1648; Hammer et al., 1987, *Science* 235:53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, *Genes and Devel.* 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, *Nature* 315:338-340; Kollias et al., 1986, *Cell* 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, *Cell* 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, *Nature* 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, *Science* 234:1372-1378).

In a specific embodiment, a vector is used that comprises a promoter operably linked to cytokine-encoding nucleic acid, one or more origins of replication, and, optionally, one or more selectable markers (*e.g.*, an antibiotic resistance gene). In another embodiment, a vector is used that comprises a promoter operably linked to ligand-encoding nucleic acid, one or more origins of replication, and, optionally, one or more selectable markers (*e.g.*, an antibiotic resistance gene). In yet another embodiment, a vector is used that comprises a promoter operably linked to a cytokine and ligand-encoding nucleic acids, one or more origins of replication, and, optionally, one or more selectable markers (*e.g.*, an antibiotic resistance gene).

Expression vectors containing gene inserts can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of the IL-12 and/or 4-1BB gene inserted in an expression vector(s) can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to the inserted gene(s). In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of the gene(s) in the vector(s).

For example, if the IL-12 gene is inserted within the marker gene sequence of the vector, recombinants containing the IL-12 gene insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the gene product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the cytokine and ligand in *in vitro* assay systems, *e.g.*, binding of IL-12 with anti-IL-12 antibody or binding of 4-1BB ligand with anti-4-1BB antibody.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (*e.g.*, lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (*e.g.*, glycosylation, phosphorylation of proteins). Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce an unglycosylated core protein product. Expression in yeast will produce a glycosylated product. Expression in mammalian cells can be used to ensure "native" glycosylation of a heterologous protein. Furthermore, different vector/host expression systems may effect processing reactions to different extents.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the differentially expressed or pathway gene protein may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which

express the differentially expressed or pathway gene protein. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the differentially expressed or pathway gene protein.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, *Cell* 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, *Proc. Natl. Acad. Sci. USA* 48:2026), and adenine phosphoribosyltransferase (Lowy et al., 1980, *Cell* 22:817) genes can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler et al., 1980, *Natl. Acad. Sci. USA* 77:3567; O'Hare et al., 1981, *Proc. Natl. Acad. Sci. USA* 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., 1981, *J. Mol. Biol.* 150:1); and hygro, which confers resistance to hygromycin (Santerre et al., 1984, *Gene* 30:147) genes.

Both cDNA and genomic sequences can be cloned and expressed.

5.2 ANTIBODIES

Antibodies of the invention include, but are not limited to, polyclonal, monoclonal, multispecific, human, humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab') fragments, fragments produced by a Fab expression library, and anti-idiotypic (anti-Id) antibodies. The term "antibody", as used herein, refers to immunoglobulin molecules and immunologically active portions thereof, *i.e.*, molecules that contain an antigen binding site that immunospecifically binds an antigen. The immunoglobulin molecules of the invention can be of any type, class, or subclass of immunoglobulin molecules. In a preferred embodiment, the immunoglobulin molecule is an antibody molecule, more preferably of a type selected from the group consisting of IgG, IgE, IgM, IgD and IgA, most preferably is an IgG molecule. In another embodiment, commercially available immunoglobulin molecules, fragments, derivatives or analogs thereof are used in the present invention. In yet another embodiment, recombinant or naturally occurring purified immunoglobulin molecules, fragments, derivatives or analogs thereof are used in the present invention.

The invention provides for the use of functionally active fragments, derivatives or analogs of the immunoglobulin molecules. Functionally active means that the fragment, derivative or analog is able to elicit anti-anti-idiotype antibodies (*i.e.*, tertiary antibodies of Ab3 antibodies) that recognize the same antigen that the antibody from which the fragment, derivative or analog is derived recognized. Specifically, in a preferred embodiment the antigenicity of the idiotype of the immunoglobulin molecule may be enhanced by deletion of framework and CDR sequences that are C-terminal to the CDR sequence that specifically

recognizes the antigen. To determine which CDR sequences bind the antigen, synthetic peptides containing the CDR sequences can be used in binding assays with the antigen by any binding assay method known in the art.

The antibodies of the invention may be from any animal origin including birds and mammals. Preferably, the antibodies are human, murine, rabbit, goat, pig, horse or chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulins and that do not express endogenous immunoglobulins (*e.g.*, the Xenomouse from Abgenix).

Antibodies of the present invention may be described in terms of their affinity to a polypeptide of the invention. Preferred binding affinities include those with a dissociation constant or Kd less than $5x10^{-6}$ M, 10^{-6} M, $5x10^{-7}$ M, 10^{-7} M, $5x10^{-8}$ M, 10^{-8} M, $5x10^{-9}$ M, 10^{-9} M, $5x10^{-10}$ M, 10^{-10} M, $5x10^{-11}$ M, 10^{-11} M, $5x10^{-12}$ M, 10^{-12} M, $5x10^{-13}$ M, 10^{-13} M, $5x10^{-14}$ M, 10^{-14} M, $5x10^{-15}$ M, or 10^{-15} M

The invention features antibodies that activate co-stimulatory molecules and cytokine receptors. The antibodies may act as receptor agonists, *i.e.*, potentiate or activate either all or a subset of the biological activities mediated by a cytokine or ligand. The activation of co-stimulatory molecules and cytokine receptors (*i.e.*, signaling) may be determined by techniques known to those of skill in the art. For example, co-stimulatory molecule and cytokine receptor activation can be determined by detecting the phosphorylation (*i.e.*, tyrosine or serine/threonine)of the molecule and receptor, respectively, or its substrate by immunoprecipitation followed by western blot analysis.

The antibodies of the invention include derivatives that are modified, *i.e.*, by the covalent attachment of any type of molecule to the antibody such that covalent attachment does not prevent the antibody from generating an anti-idiotypic response. For example, but not by way of limitation, the antibody derivatives include antibodies that have been modified, *e.g.*, by glycosylation, acetylation, pegylation, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to, specific chemical cleavage, acetylation, formylation, and metabolic synthesis of tunicamycin. Additional the derivative may contain one or more non-classical amino acids.

The present invention encompasses antibodies recombinantly fused or chemically conjugated (including both covalently and non-covalently conjugations) to a polypeptide (or portion thereof, preferably at least 10, 20 or 50 amino acids of the polypeptide) of the present invention to generate fusion proteins. The fusion does not necessarily need to be direct, but may occur through linker sequences. The antibodies may be specific for antigens other than polypeptides (or portion thereof, preferably at least 10, 20 or 50 amino acids of the polypeptide) of the present invention.

The present invention encompasses antibodies conjugated to a therapeutic moiety such as a cytotoxin, *e.g.*, a cytostatic or cytocidal agent, a therapeutic agent or a radioactive metal ion. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include paclitaxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (*e.g.*, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (*e.g.*, mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (*e.g.*, daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (*e.g.*, dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (*e.g.*, vincristine and vinblastine).

The conjugates of the invention can be used for modifying a given biological response, and the therapeutic agent or drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor, α-interferon, β-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator, a thrombotic agent or an anti-angiogenic agent, *e.g.*, angiostatin or endostatin; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophase colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

Techniques for conjugating a therapeutic moiety to antibodies are well known, see, *e.g.*, Arnon *et al.*, "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld *et al.* (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom *et al.*, "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson *et al.* (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera *et al.* (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin *et al.* (eds.), pp. 303-16 (Academic Press 1985), and Thorpe *et al.*, "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", *Immunol. Rev.* 62:119-58 (1982).

The antibodies of the present invention may be generated by any suitable method known in the art. Polyclonal antibodies to an antigen-of-interest can be produced by various procedures well known in the art. For example, a polypeptide of the invention can be administered to various host animals including, but not limited to, rabbits, mice, rats, etc. to induce the production of sera containing polyclonal antibodies specific for the antigen. Various adjuvants may be used to increase the immunological response, depending on the host species, and include but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum. Such adjuvants are also well known in the art.

Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow *et al.*, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling, *et al.*, in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981) (said references incorporated by reference in their entireties). The term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. The term "monoclonal antibody" refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced.

Methods for producing and screening for specific antibodies using hybridoma technology are routine and well known in the art. Briefly, mice can be immunized with a polypeptide of the invention or a cell expressing such peptide. Once an immune response is detected, *e.g.*, antibodies specific for the antigen are detected in the mouse serum, the mouse spleen is harvested and splenocytes isolated. The splenocytes are then fused by well known techniques to any suitable myeloma cells, for example cells from cell line SP20 available from the ATCC. Hybridomas are selected and cloned by limited dilution. The hybridoma clones are then assayed by methods known in the art for cells that secrete antibodies capable of binding a polypeptide of the invention. Ascites fluid, which generally contains high levels of antibodies, can be generated by immunizing mice with positive hybridoma clones.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, Fab and F(ab')2 fragments of the invention may be produced by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')2 fragments). F(ab')2 fragments contain the variable region, the light chain constant region and the CH1 domain of the heavy chain.

The antibodies of the present invention may also be generated using various phage

display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of a phage particles which carry the polynucleotide sequences encoding them. In a particular, such phage can be utilized to display antigen binding domains expressed from a repertoire or combinatorial antibody library (*e.g.*, human or murine). Phage expressing an antigen binding domain that binds the antigen of interest can be selected or identified with antigen, *e.g.*, using labeled antigen or antigen bound or captured to a solid surface or bead. Phage used in these methods are typically filamentous phage including fd and M13 binding domains expressed from phage with Fab, Fv or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein. Examples of phage display methods that can be used to make the antibodies of the present invention include those disclosed in Brinkman *et al.*, *J. Immunol. Methods* 182:41-50 (1995); Ames *et al.*, *J. Immunol. Methods* 184:177-186 (1995); Kettleborough *et al.*, *Eur. J. Immunol.* 24:952-958 (1994); Persic *et al.*, *Gene* 187 9-18 (1997); Burton *et al.*, *Advances in Immunology* 57:191-280 (1994); PCT application No. PCT/GB91/01134; PCT publications WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and U.S. Patent Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108; each of which is incorporated herein by reference in its entirety.

Examples of techniques which can be used to produce single-chain Fvs and antibodies include those described in U.S. Patents 4,946,778 and 5,258,498; Huston *et al.*, *Methods in Enzymology* 203:46-88 (1991); Shu *et al.*, *PNAS* 90:7995-7999 (1993); and Skerra *et al.*, *Science* 240:1038-1040 (1988). For some uses, including *in vivo* use of antibodies in humans and *in vitro* detection assays, it may be preferable to use chimeric, humanized, or human antibodies. A chimeric antibody is a molecule in which different portions of the antibody are derived from different animal species, such as antibodies having a variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region. Methods for producing chimeric antibodies are known in the art. See *e.g.*, Morrison, *Science* 229:1202 (1985); Oi *et al.*, *BioTechniques* 4:214 (1986); Gillies *et al.*, (1989) *J. Immunol. Methods* 125:191-202; U.S. Patent Nos. 5,807,715; 4,816,567; and 4,816,397, which are incorporated herein by reference in their entirety. Humanized antibodies are antibody molecules from non-human species antibody that binds the desired antigen having one or more complementarity determining regions (CDRs) from the non-human species and a framework regions from a human immunoglobulin molecule. Often, framework residues in the human framework regions will be substituted with the corresponding residue from the CDR donor antibody to alter, preferably improve, antigen binding. These framework substitutions are identified by methods well known in the art, *e.g.*, by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. (See,

e.g., Queen *et al.*, U.S. Patent No. 5,585,089; Riechmann *et al.*, *Nature* 332:323 (1988), which are incorporated herein by reference in their entireties.) Antibodies can be humanized using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; PCT publication WO 91/09967; U.S. Patent Nos. 5,225,539; 5,530,101; and 5,585,089), veneering or resurfacing (EP 592,106; EP 519,596; Padlan, *Molecular Immunology* 28(4/5):489-498 (1991); Studnicka *et al.*, *Protein Engineering* 7(6):805-814 (1994); Roguska. *et al.*, *PNAS* 91:969-973 (1994)), and chain shuffling (U.S. Patent No. 5,565,332).

Completely human antibodies are particularly desirable for therapeutic treatment of human patients. Human antibodies can be made by a variety of methods known in the art including phage display methods described above using antibody libraries derived from human immunoglobulin sequences. See also, U.S. Patent Nos. 4,444,887 and 4,716,111; and PCT publications WO 98/46645, WO 98/50433, WO 98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741; each of which is incorporated herein by reference in its entirety.

Human antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable region, constant region, and diversity region may be introduced into mouse embryonic stem cells in addition to the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous deletion of the J_H region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then be bred to produce homozygous offspring which express human antibodies. The transgenic mice are immunized in the normal fashion with a selected antigen, *e.g.*, all or a portion of a polypeptide of the invention. Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg and Huszar (1995, *Int. Rev. Immunol.* 13:65-93). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, *see, e.g.*, PCT publications WO 98/24893; WO 96/34096; WO 96/33735; U.S. Patent Nos. 5,413,923; 5,625,126; 5,633,425;

5,569,825; 5,661,016; 5,545,806; 5,814,318; and 5,939,598, which are incorporated by reference herein in their entirety. In addition, companies such as Abgenix, Inc. (Freemont, CA) and Genpharm (San Jose, CA) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

5.3 METHOD OF PRODUCING ANTIBODIES

The antibodies of the invention can be produced by any method known in the art for the synthesis of immunoglobulins, in particular, by chemical synthesis or by recombinant expression, and is preferably produced by recombinant expression techniques.

Recombinant expression of an immunoglobulin molecule of the invention, or fragment, derivative or analog thereof, requires construction of a nucleic acid that encodes the immunoglobulin. If the nucleotide sequence of the immunoglobulin is known, a nucleic acid encoding the immunoglobulin may be assembled from chemically synthesized oligonucleotides (*e.g.*, as described in Kutmeier et al., 1994, *BioTechniques* 17:242), which, briefly, involves the synthesis of overlapping oligonucleotides containing portions of the sequence encoding the immunoglobulin, annealing and ligation of those oligonucleotides, and then amplification of the ligated oligonucleotides by PCR.

Alternatively, the nucleic acid encoding the immunoglobulin may be generated from a nucleic acid encoding the immunoglobulin. If a clone containing the nucleic acid encoding the particular immunoglobulin is not available, but the sequence of the immunoglobulin molecule is known, a nucleic acid encoding the immunoglobulin may be obtained from a suitable source (*e.g.*, an antibody cDNA library, or cDNA library generated from any tissue or cells expressing the immunoglobulin) by PCR amplification using synthetic primers hybridizable to the 3' and 5' ends of the sequence or by cloning using an oligonucleotide probe specific for the particular gene sequence.

In an immunoglobulin molecule that specifically recognizes a particular antigen is not available (or a source for a cDNA library for cloning a nucleic acid encoding such an immunoglobulin), immunoglobulins specific for a particular antigen may be generated by any method known in the art, for example, by immunizing an animal, such as a rabbit, to generate polyclonal antibodies or, more preferably, by generating monoclonal antibodies, *e.g.*, as described by Kohler and Milstein (1975, *Nature* 256:495-497) or, as described by Kozbon et al. (1983, *Immunology Today* 4:72) or Cole et al. (1985 in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96). Alternatively, a clone encoding at least the Fab portion of the immunoglobulin by screening Fab expression libraries (*e.g.*, as described in Huse et al., 1989, *Science* 246:1275-1281) for clones of Fab fragments that bind the specific antigen or by screening antibody libraries (See, *e.g.*, Clackson et al., 191, Nature 352:624; Hane et al., 1997 Proc. Natl. Acad. Sci. USA 94:4937).

Once a nucleic acid encoding at least the variable domain of the immunoglobulin molecule is obtained, it may be introduced into a vector containing the nucleotide sequence encoding the constant region of the immunoglobulin molecule (see, *e.g.*, PCT Publication WO 86/05807; PCT Publication WO 89/01036; and U.S. Patent No. 5,122,464). Vectors containing the complete light or heavy chain for co-expression with the nucleic acid to allow the expression of a complete antibody molecule are also available. Then, the nucleic acid encoding the immunoglobulin can be to introduce the nucleotide substitutions or deletion necessary to substitute (or delete) the one or more variable region cysteine residues participating in an intrachain disulfide bond with an amino acid residue that does not contain a sulfhydyl group. Such modifications can be carried out by any method known in the art for the introduction of specific mutations or deletions in a nucleotide sequence, for example, but not limited to, chemical mutagenesis, *in vitro* site directed mutagenesis (Hutchinson et al., 1978, *J. Biol. Chem.* 253:6551), PCT based methods, etc.

Once a nucleic acid encoding the immunoglobulin molecule of the invention has been obtained, the vector for the production of the immunoglobulin molecule may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing the protein of the invention by expressing nucleic acid containing the immunoglobulin molecule sequences are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing the immunoglobulin molecule coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. See, for example, the techniques described in Sambrook et al. (1990, *Molecular Cloning, A Laboratory Manual*, 2d Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and Ausubel et al. (eds., 1998, *Current Protocols in Molecular Biology*, John Wiley & Sons, NY).

The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce the immunoglobulin of the invention.

The host cells used to express the recombinant antibody of the invention may be either bacterial cells such as *Escherichia coli,* or, preferably, eukaryotic cells, especially for the expression of whole recombinant immunoglobulin molecule. In particular, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system for immunoglobulins (Foecking et al., 198, *Gene* 45:101; Cockett et al., 1990, *Bio/Technology* 8:2).

A variety of host-expression vector systems may be utilized to express the immunoglobulin molecules of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently

purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express the immunoglobulin molecule of the invention in situ. These include but are not limited to microorganisms such as bacteria (*e.g.*, *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing immunoglobulin coding sequences; yeast (*e.g.*, *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing immunoglobulin coding sequences; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the immunoglobulin coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing immunoglobulin coding sequences; or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the immunoglobulin molecule being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of an immunoglobulin molecule, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther et al., 1983, *EMBO J.* 2:1791), in which the immunoglobulin coding sequence may be ligated individually into the vector in frame with the *lac Z* coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, *Nucleic Acids Res.* 13:3101-3109; Van Heeke & Schuster, 1989, *J. Biol. Chem.* 24:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to a matrix glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The immunoglobulin coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the immunoglobulin coding

sequence of interest may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the immunoglobulin molecule in infected hosts. (*e.g.*, See Logan & Shenk, 1984, *Proc. Natl. Acad. Sci. USA* 81:355-359). Specific initiation signals may also be required for efficient translation of inserted immunoglobulin coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, *Methods in Enzymol.* 153:51-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and in particular, breast cancer cell lines such as, for example, BT483, Hs578T, HTB2, BT20 and T47D, and normal mammary gland cell line such as, for example, CRL7030 and Hs578Bst.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the immunoglobulin molecule may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the

immunoglobulin molecule. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that interact directly or indirectly with the immunoglobulin molecule.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al., 1977, *Cell* 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 192, *Proc. Natl. Acad. Sci. USA* 48:202), and adenine phosphoribosyltransferase (Lowy et al., 1980, *Cell* 22:817) genes can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler et al., 1980, *Natl. Acad. Sci. USA* 77:357; O'Hare et al., 1981, *Proc. Natl. Acad. Sci. USA* 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78:2072); neo, which confers resistance to the aminoglycoside G-418Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; and Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; May, 1993, TIB TECH 11(5):155-215). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY; and in Chapters 12 and 13, Dracopoli et al. (eds), 1994, Current Protocols in Human Genetics, John Wiley & Sons, NY.; Colberre-Garapin et al., 1981, *J. Mol. Biol.* 150:1; and hygro, which confers resistance to hygromycin (Santerre et al., 1984, *Gene* 30:147).

Alternatively, any fusion protein may be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht et al. allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht et al., 1991, *Proc. Natl. Acad. Sci. USA* 88:8972-897). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the open reading frame of the gene is translationally fused to an amino-terminal tag consisting of six histidine residues. The tag serves as a matrix binding domain for the fusion protein. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni^{2+}-nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

The expression levels of the immunoglobulin molecule can be increased by vector amplification (for a review, see Bebbington and Hentschel, *The use of vectors based on gene amplification for the expression of cloned genes in mammalian cells in DNA cloning*, Vol.3. (Academic Press, New York, 1987)). When a marker in the vector system expressing immunoglobulin is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is

associated with the immunoglobulin gene, production of the immunoglobulin will also increase (Crouse et al., 1983, *Mol. Cell. Biol.* 3:257).

The host cell may be co-transfected with two expression vectors of the invention, the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes both heavy and light chain polypeptides. In such situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot, 1986, *Nature* 322:52; Kohler, 1980, *Proc. Natl. Acad. Sci. USA* 77:2197). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

Once the immunoglobulin molecule of the invention has been recombinantly expressed, it may be purified by any method known in the art for purification of an immunoglobulin molecule, for example, by chromatography (*e.g.*, ion exchange, affinity, particularly by affinity for the specific antigen after Protein A, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

5.4 THERAPEUTIC USES OF COMPOSTIONS

The invention provides for treatment, prevention or inhibition of diseases and disorders, including cancer, inflammatory diseases and infectious diseases by administration of a therapeutic or pharmaceutical composition. In a preferred embodiment, pharmaceutical compositions are administered to a subject (*i.e.*, an animal) to treat, prevent or inhibit cancer. Examples of types of cancer, include, but are not limited to, leukemia (*e.g.*, acute leukemia such as acute lymphocytic leukemia and acute myelcytic leukemia), neoplasms, tumors (*e.g.*, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, and retinoblastoma), heavy chain disease, metastases, or any disease or disorder characterized by uncontrolled cell growth.

In another embodiment, pharmaceutical compositions are administered to a subject (*i.e.*, an animal) to treat, prevent or inhibit inflammatory diseases. Examples of inflammatory diseases include, but are not limited to, systemic lupus erythematosus, rheumatoid arthritis, acute respiratory distress syndrome, asthma, and osteoporosis). In another embodiment, pharmaceutical compositions are administered to a subject (*i.e.*, an animal) to treat, prevent or inhibit infectious diseases. Infectious diseases include diseases associated with yeast, fungal, viral and bacterial infections. Viruses causing viral infections include, but are limited to, herpes simplex virus (HSV), hepatitis B virus (HBV), hepatitis C virus (HCV), human T-cell lymphotrophic virus (HTLV) type I and II, human immunodeficiency virus (HIV), cytomegalovirus, papilloma virus, polyoma viruses, adenoviruses, Epstein-Barr virus, poxviruses, influenza virus, measles virus, rabies virus, Sendai virus, poliomyelitis virus, coxsackieviruses, rhinoviruses, reoviruses, and rubella virus. Microbial pathogens causing bacterial infections include, but are not limited to, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Neisseria gonorrhoea*, *Neisseria meningitidis*, *Corynebacterium diphtheriae* , *Clostridium botulinum*, *Clostridium perfringens*, *Clostridium tetani*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Klebsiella ozaenae*, *Klebsiella rhinoscleromotis*, *Staphylococcus aureus*, *Vibrio cholerae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Campylobacter* (Vibrio) *fetus*, *Campylobacter jejuni*, *Aeromonas hydrophila*, *Bacillus cereus*, *Edwardsiella tarda*, *Yersinia enterocolitica*, *Yersinia pestis*, *Yersinia pseudotuberculosis*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, *Salmonella typhimurium*, *Treponema pallidum*, *Treponema pertenue*, *Treponema carateneum*, *Borrelia vincentii*, *Borrelia burgdorferi*, *Leptospira icterohemorrhagiae*, *Mycobacterium tuberculosis*, *Toxoplasma gondii*, *Pneumocystis carinii*, *Francisella tularensis*, *Brucella abortus*, *Brucella suis*, *Brucella melitensis*, *Mycoplasma spp.*, *Rickettsia prowazeki*, *Rickettsia tsutsugumushi*, *Chlamydia spp.*, and *Helicobacter pylori*.

In specific embodiments, a subject is administered a composition of the present invention in an amount effective for the treatment, prevention or inhibition of a disease or disorder such as cancer and an infectious disease, or an amount effective for inducing an anti-tumor response (*e.g.*, the inhibition of the hyperproliferation of a tumor), or an amount effective for augmenting activated immune cells, or an amount effective such that the immune response in a subject (an animal) is augmented.

Diseases and disorders described herein may be treated, prevented or inhibited by administration of therapeutic composition (*i.e.*, a combination of a cytokine and an NK and/or T-cell activating molecule) and that the augments activated NK and T-cells. In particular, therapeutic and pharmaceutical compositions of the present invention comprise: (i) one or more cytokines in combination with one or more ligands for co-stimulatory molecules expressed on activated immune cells (*i.e.*, NK cells and T-cells); (ii) one or more cytokines in combination with antibodies which bind to and activate co-stimulatory molecules expressed

on activated immune cells (*i.e.*, NK cells and T-cells); (iii) antibodies which interact and activate cytokine receptors in combination with one or more ligands for co-stimulatory molecules expressed on activated immune cells (*i.e.*, NK cells and T-cells); and (iv) antibodies which bind to and activate co-stimulatory molecules expressed on activated immune cells (*i.e.*, NK cells and T-cells) in combination with antibodies which bind to and activate cytokine receptors. The polypeptides can be supplied by direct administration or indirectly as "pro-drugs" using somatic cell gene therapy.

In one embodiment, therapeutic or pharmaceutical composition comprising: (i) native or recombinant IL-12 polypeptides, peptides, fragments, derivatives or analogs thereof in combination with native or recombinant 4-1BBL polypeptides, peptides, fragments, derivatives or analogs thereof; (ii) native or recombinant IL-12 polypeptides, peptides, fragments, derivatives or analogs thereof in combination with anti-4-1BB antibodies, (iii) anti-IL-2 receptor (IL-12R) antibodies in combination with native or recombinant 4-1BBL polypeptides, peptides, fragments, derivatives or analogs thereof; and (iv) anti-IL-12R antibodies in combination with anti-4-1BB antibodies. The proteins can be supplied by direct administration or indirectly as "pro-drugs" using somatic cell gene therapy. Commercially available or naturally occurring proteins, functionally active fragments or derivatives of 4-1BB ligand or anti-4-1BB antibody and IL-12 may be used in the present invention.

Generally, administration of products autologous to the patient is preferred. Thus, in preferred embodiment, human 4-1BB ligand or anti-41BB antibody and IL-12, derivatives, or analogs, or nucleic acids, or are administered to a human patient for therapy or prophylaxis.

5.4.1 GENE THERAPY

In one embodiment, nucleic acid molecules comprising sequences encoding one or more cytokines and ligands for co-stimulatory molecules expressed on activated immune cells (*i.e.*, NK and T-cells) are administered to promote T-cell and NK cell function, by way of gene therapy. In another embodiment, nucleic acid molecules comprising sequences encoding one or more cytokines and antibodies which bind to and activate co-stimulatory molecules expressed on activated immune cells (*i.e.*, NK and T-cells) are administered to promote T-cell and NK cell function, by way of gene therapy. In another embodiment, nucleic acid molecules comprising sequences encoding antibodies which bind to and activate cytokine receptors and ligands for co-stimulatory molecules expressed on activated immune cells (*i.e.*, NK and T-cells) are administered to promote T-cell and NK cell function, by way of gene therapy. In yet another embodiment, nucleic acid molecules comprising sequences encoding antibodies which bind to and activate cytokine receptors and antibodies which bind to and activate co-stimulatory molecules expressed on activated immune cells (*i.e.*, NK and T-cells) are administered to promote T-cell and NK cell function, by way of gene therapy. In a preferred embodiment, nucleic acid sequences encoding 4-1BB ligand or anti-4-1BB

immunoglobulins and IL-12 or functional derivatives thereof, are administered to augment the function (*i.e.*, proliferation and differentiation) of activated T-cell and NK cells, by way of gene therapy. Gene therapy refers to therapy performed by the administration to a subject of an expressed or expressible nucleic acid.

Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, see Goldspiel et al., 1993, *Clinical Pharmacy* 12:488-505; Wu and Wu, 1991, *Biotherapy* 3:87-95; Tolstoshev, 1993, *Ann. Rev. Pharmacol. Toxicol.* 32:573-596; Mulligan, 1993, *Science* 260:926-932; and Morgan and Anderson, 1993, *Ann. Rev. Biochem.* 62:191-217; May, 1993, TIBTECH 11(5):155-215). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; and Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY.

In a preferred aspect, a composition of the invention comprises nucleic acid sequences encoding 4-1BB ligand or anti-4-1BB immunoglobulin and IL-12, said nucleic acid sequences being part of expression vectors that express 4-1BB ligand or anti-4-1BB immunoglobulin and IL-12 or fragments or chimeric proteins thereof in a suitable host. In particular, such nucleic acid sequences have promoters operably linked to the 4-1BB ligand or anti-4-1BB immunoglobulin and IL-12 coding region, said promoter being inducible or constitutive, and, optionally, tissue-specific. In another particular embodiment, nucleic acid molecules are used in which the 4-1BB ligand or anti-4-1BB immunoglobulin and IL-12 coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the 4-1BB ligand or anti-4-1BB immunoglobulin and IL-12 nucleic acids (Koller and Smithies, 1989, *Proc. Natl. Acad. Sci. USA* 86:8932-8935; Zijlstra et al., 1989, *Nature* 342:435-438).

Delivery of the nucleic acids into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vectors, or indirect, in which case, cells are first transformed with the nucleic acids *in vitro*, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy.

In a specific embodiment, the nucleic acid sequences are directly administered *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, *e.g.*, by constructing them as part of an appropriate nucleic acid expression vector and administering it so that they become intracellular, *e.g.*, by infection using defective or attenuated retrovirals or other viral vectors (see U.S. Patent No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or

transfecting agents, encapsulation in liposomes, microparticles, or microcapsules, or by administering them in linkage to a peptide which is known to enter the nucleus, by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, 1987, *J. Biol. Chem.* 262:4429-4432) (which can be used to target cell types specifically expressing the receptors), etc. In another embodiment, nucleic acid-ligand complexes can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (see, *e.g.*, PCT Publications WO 92/06180 dated April 16, 1992 (Wu et al.); WO 92/22635 dated December 23, 1992 (Wilson et al.); WO92/20316 dated November 26, 1992 (Findeis et al.); WO93/14188 dated July 22, 1993 (Clarke et al.), WO 93/20221 dated October 14, 1993 (Young)). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, 1989, *Proc. Natl. Acad. Sci. USA* 86:8932-8935; Zijlstra et al., 1989, *Nature* 342:435-438).

In one embodiment, viral vectors that contain nucleic acids encoding compounds that activate cytokine receptors (*i.e.*, cytokines and antibodies) and compounds that activate co-stimulatory molecules expressed on activated immune cells (*i.e.*, ligands and antibodies) are used (see Miller et al., 1993, *Meth. Enzymol.* 217:581-599). In a specific embodiment, viral vectors that contains nucleic acid sequences encoding 4-1BB ligand or anti-4-1BB immunoglobulin and IL-12 are used. For example, a retroviral vector can be used. These retroviral vectors have been to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA. The nucleic acid sequences encoding the 4-1BB ligand or anti-4-1BB immunoglobulin and IL-12 to be used in gene therapy are cloned into one or more vectors, which facilitates delivery of the gene into a patient. More detail about retroviral vectors can be found in Boesen et al., 1994, *Biotherapy* 6:291-302, which describes the use of a retroviral vector to deliver the mdr1 gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes et al., 1994, *J. Clin. Invest.* 93:644-651; Kiem et al., 1994, *Blood* 83:1467-1473; Salmons and Gunzberg, 1993, *Human Gene Therapy* 4:129-141; and Grossman and Wilson, 1993, *Curr. Opin. in Genetics and Devel.* 3:110-114.

Adenoviruses are other viral vectors that can be used in gene therapy. Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, 1993, *Current Opinion in Genetics and Development* 3:499-503

present a review of adenovirus-based gene therapy. Bout et al., 1994, *Human Gene Therapy* 5:3-10 demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld et al., 1991, *Science* 252:431-434; Rosenfeld et al., 1992, *Cell* 68:143-155; Mastrangeli et al., 1993, *J. Clin. Invest.* 91:225-234; PCT Publication WO94/12649; and Wang, et al., 1995, *Gene Therapy* 2:775-783. In a preferred embodiment, adenovirus vectors are used.

Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh et al., 1993, *Proc. Soc. Exp. Biol. Med.* 204:289-300; U.S. Patent No. 5,436,146).

Another approach to gene therapy involves transferring a gene to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a patient.

In this embodiment, the nucleic acid is introduced into a cell prior to administration *in vivo* of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction of foreign genes into cells (see, *e.g.*, Loeffler and Behr, 1993, *Meth. Enzymol.* 217:599-618; Cohen et al., 1993, *Meth. Enzymol.* 217:618-644; Cline, 1985, *Pharmac. Ther.* 29:69-92) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a patient by various methods known in the art. Recombinant blood cells (*e.g.*, hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, patient state, etc., and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, *e.g.*, as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, etc.

In a preferred embodiment, the cell used for gene therapy is autologous to the patient.

In one embodiment in which recombinant cells are used in gene therapy, nucleic acid sequences encoding compounds that activate cytokine receptors (*i.e.*, cytokines and antibodies) and compounds that activate co-stimulatory molecules expressed on activated immune cells (*i.e.*, ligands and antibodies) are introduced into the cells such that they are expressible by the cells or their progeny, and the recombinant cells are then administered *in vivo* for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem and/or progenitor cells which can be isolated and maintained *in vitro* can potentially be used in accordance with this embodiment of the present invention (see e.g. PCT Publication WO 94/08598, dated April 28, 1994; Stemple and Anderson, 1992, *Cell* 71:973-985; Rheinwald, 1980, *Meth. Cell Bio.* 21A:229; and Pittelkow and Scott, 1986, *Mayo Clinic Proc.* 61:771).

In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription.

5.5 DEMONSTRATION OF THERAPEUTIC OR PROPHYLACTIC UTILITY

The compounds of the invention are preferably tested *in vitro*, and then *in vivo* for the desired therapeutic or prophylactic activity, prior to use in humans. For example, *in vitro* assays which can be used to determine whether administration of a specific compound is indicated, include *in vitro* cell culture assays in which a patient tissue sample is grown in culture, and exposed to or otherwise administered a compound, and the effect of such compound upon the tissue sample is observed.

Test compounds can be tested for their ability to augment activated immune cells by contacting activated immune cells with a test compound or a control compound and determining the ability of the test compound to modulate (*e.g.*, increase) the biological activity of the activated immune cells. The ability of a test compound to modulate the biological activity of activated immune cells can be assessed by detecting the expression of cytokines or antigens, detecting the proliferation of immune cells, detecting the activation of signaling molecules, detecting the effector function of immune cells, or detecting the differentiation of immune cells. Techniques known to those of skill in the art can be used for measuring these activities. For example, cellular proliferation can be assayed by ^{3}H-thymidine incorporation assays and trypan blue cell counts. Cytokine and antigen expression can be assayed, for example, by immunoassays including, but are not limited to, competitive and non-competitive assay systems using techniques such as western blots, immunohistochemistry radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin

reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays and FACS analysis. The activation of signaling molecules can be assayed, for example, by kinase assays and electromobility shift assays (EMSAs). The effector function of T-cells can be measured, for example, by a ^{51}Cr-release assay (see, *e.g.*, Palladino et al., 1987, Cancer Res. 47:5074--5079 and Blachere et al., 1993, J. Immunotherapy 14:352-356).

Test compounds can be tested for their ability to reduce tumor formation in patients (*i.e.*, animals) suffering from cancer. Test compounds can also be tested for their ability to reduce viral load or bacterial numbers patients suffering from an infectious disease. Test compounds can also be tested for their ability to alleviate of one or more symptoms associated with cancer or an infectious disease. Test compounds can also be tested for their ability to decrease the time course of the infectious disease. Further, test compounds can be tested for their ability to increase the survival period of patients suffering from cancer or an infectious disease. Techniques known to those of skill in the art can be used to analyze test to function of the test compounds in patients.

In various specific embodiments, *in vitro* assays can be carried out with representative cells of cell types involved in a patient's disorder, to determine if a compound has a desired effect upon such cell types.

Compounds for use in therapy can be tested in suitable animal model systems prior to testing in humans, including but not limited to rats, mice, chicken, cows, monkeys, rabbits, etc. For *in vivo* testing, prior to administration to humans, any animal model system known in the art may be used.

5.6 THERAPEUTIC/PROPHYLACTIC ADMINISTRATION AND COMPOSITIONS

The invention provides methods of treatment (and prophylaxis) by administration to a subject of an effective amount of a compound of the invention. In a preferred aspect, the compound is substantially purified (*e.g.*, substantially free from substances that limit its effect or produce undesired side-effects). The subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human. In a specific embodiment, a non-human mammal is the subject.

Formulations and methods of administration that can be employed when the compound comprises a nucleic acid are described above; additional appropriate formulations and routes of administration can be selected from among those described herein below.

Various delivery systems are known and can be used to administer a compound of the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (see, *e.g.*, Wu and Wu,

1987, *J. Biol. Chem.* 262:4429-4432), construction of a nucleic acid as part of a retroviral or other vector, etc. Methods of introduction include but are not limited to intratumoral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, *e.g.*, in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a malignant tumor or neoplastic or pre-neoplastic tissue.

In another embodiment, the compound can be delivered in a vesicle, in particular a liposome (*see* Langer, *Science* 249:1527-1533 (1990); Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; *see* generally *ibid.*)

In yet another embodiment, the compound can be delivered in a controlled release system. In one embodiment, a pump may be used (*see* Langer, *supra*; Sefton, *CRC Crit. Ref. Biomed. Eng.* 14:201 (1987); Buchwald et al., *Surgery* 88:507 (1980); Saudek et al., *N. Engl. J. Med.* 321:574 (1989)). In another embodiment, polymeric materials can be used (*see* Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, *J. Macromol. Sci. Rev. Macromol. Chem.* 23:61 (1983); *see also* Levy et al., *Science* 228:190 (1985); During et al., *Ann. Neurol.* 25:351 (1989); Howard et al., *J. Neurosurg.* 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, *i.e.*, the brain, thus requiring only a fraction of the systemic dose (*see, e.g.*, Goodson, in Medical Applications of Controlled Release, *supra*, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (*Science* 249:1527-1533 (1990)).

In a specific embodiment where the compound of the invention is a nucleic acid encoding a protein, the nucleic acid can be administered *in vivo* to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see *e.g.*, Joliot et al., 1991, *Proc. Natl. Acad. Sci. USA* 88:1864-1868), etc. Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a compound, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human

beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The compounds of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the compound of the invention which will be effective in the treatment of prostate cancer can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for intravenous administration are generally about 20-500 micrograms of active compound per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

6. EXAMPLE: REJECTION OF HEPATIC COLON CARCINOMA AND LUNG METASTASES BY IMMUNOMODULATORY THERAPY WITH 4-1BB LIGAND OR ANTI-4-1BB AND IL-12

The effectiveness of therpeuctic compositions comprising IL-12 in combination with 4-1BB ligand or anti-4-1BB in the long-term survival of animal models of liver and macroscopic lung metastatic tumors were evaluated according to the experimental design described below.

6.1 MATERIALS AND METHODS

Tumor model and therapeutic protocols

MCA26 is a tumor cell line of chemically induced colon carcinoma in BALB/c mouse (Corbett et al., 1975). Metastatic colon cancer was induced by implanting $7x10^4$ MCA26 cells into the left lobe of the liver of 8-10 week old female BALB/c mice (Taconic). At day 7, mice with 5x5 imm^2 size tumors were selected and different dose of Adv.mIL-12 or control DL312 vector were injected intratumorally in a 50µl volume of 10mM Tris-HCl (pH 7.4)/1 mM MgC12/10% (vol/vol) glycerol/Polybrene (20 µg/ml). At day 8 and day 11, 50µg anti-4-1BB antibody or control rat Ig was administered intraperitoneally (i.p.).

JC cell line is a chemically induced breast carcinoma line derived from a BALB/c background. JC cells are grown and maintained in MEM supplemented with 10% fetal calf serum, 2mM L-glutamine, 100-unit/ml penicillin, and 100-mg/ml streptomycin. Using the same model of liver metastases as previously described, the left lateral lobe of the liver of adult inbred female BALB/c mice (8- to 10-week old, 18- to 20-g) was injected directly with $1x10^5$ JC cells suspended in 10-µl Hank's balanced salt solution. Ten days after injection, liver tumors measuring between 5x5 mm in diameter were directly injected with ADV/IL-12 or a control vector ADV/DL312 ($1x10^8$ pfu/animal). Animals attributed to the gene therapy group received intra-tumoral delivery of ADV/4-1BBL or ADV/DL312 ($1x10^9$ pfu/animal) in combination to ADV/IL-12. If assigned to the antibody treatment group, 50 µg of monoclonal anti-4-1BB agonistic antibody or control rat IgG were injected i.p. on day one and three after intratumoral gene treatment with ADV/IL-12. Long-term survival studies were performed to assess treatment outcome.

Rechallenge test and *in vivo* depletion of lymphocytes

About 100 days after the treatment, the original tumors in survival mice were eradicated. The rechallenge test was performed by implanting $7x10^4$ MCA26 cells subcutaneously on the shaved flanks of the survival animals. Alternatively, the rechallenge test was performed by implanting JC parental tumor cells ($1x10^5$) and MCA26 cells ($7x10^4$) subcutaneously (s.c.) on left and right flanks, respectively, of mice that survived long-term (>120 days) after ADV/IL-12 plus ADV/4-1BBL, ADV/IL-12 + anti-4-1BB, ADV/IL-12 or

anti-4-1BB alone treatment., respectively. Animals were observed for tumor formation and rate of tumor growth. "Naive" BALB/c mice that have never been exposed to JC or MCA26 cells were used to assess the normal growth of a s.c. JC or MCA26 tumor.

For *in vivo* depletion of T-cells (CD8+) or NK cells, either purified ascites from 2.43 hybridoma (ATCC) or polyclonal antibodies anti-asialo GM1 (Wako Co.) or appropriate Ig controls was injected intraperitoneally under established procedures (Brunda et al., 1993, *J. Leukocyte Biology* 55: 280-288; Colombo et al., 1996, Cancer Res. 56: 2531-2534; Nishmura et al., 1995, *Immunology Letters* 48: 167-174; Scott 1993, *Science* 260: 496-497; and Takeda et al., 1996, *J. Immunology* 156: 3366-3373). The mice were given 2 mg of antibody i.p. per day, beginning one day prior to tumor rechallenge. Antibodies for control and NK+ depletion were administered for five consecutive days then every five days afterward (day -1, 0, 1, 2, 3, 8, 13), while antibodies for CD8+ depletion were given on every other day for three times and then every five days afterward (day -1, 1, 3, 8, 13) according to established optimal conditions. Treatment efficiencies with these antibodies were confirmed by flow cytometry, and effectively depleted subsets (>99%) of the immune lymphocytes were routinely obtained.

Construction of the IL-12 virus vector

A recombinant adenovirus expressing mIL-12 was constructed by replacing the E1A region of adenovirus type 5 with an expression cassette pAd/RSV-mIL-12 containing two IL-12 cDNA subunits, p35 and p40, linked by an internal ribosomal entry site (IRES) of the encephalomyocarditis virus (Banks et al., 1995, *Br. J. Cancer* 71: 655-659; Brunda 1994, *J. Leukocyte Biology* 55: 280-288; Brunda et al., 1993, *J. Exp. Med.* 178:1223-1230; Caruso et al., 1996, *Proc. Natl. Acad. Sci., USA* 93: 11302-11306; Chen et al., 1997, *J. Immunology* 159: 351-359; and Tahara et al., 1995, *J. Immunology* 154: 6466-6474). The recombinant virus was generated by cotransfection with pAd/RSV-mIL-12 and pBHG10 into 293 cells using calcium phosphate precipitation method. Large scale production of recombinant adenovirus was accomplished in 293 cells and purified by double cesium chloride gradient ultracentrifugation. The viral titer [plaque forming units (pfu)/ml] was determined by plaque assay in the 293 cells (Caruso et al., 1996, *Proc. Natl. Acad. Sci., USA* 93: 11302-11306; and Chen et al., 1997, *J. Immunology* 159: 351-359). Bioactivity was determined by ELISA for the IFNγ release from naive mouse splenocytes cocultured with supernatant from Adv.mIL-12-transduced (1000 m.o.i.) MCA26 tumor cells.

Construction and Characterization of the recombinant adenoviral vector expressing 4-1BB ligand

The full-length mouse 4-1BB ligand cDNA was obtained from pLXSHDm41BB-ligand by PCR amplification using appropriate primers with EcoR V and Not I linkers. The cDNA clone with the correct sequence was subcloned into the adenoviral backbone vector

(pAdl.1/RSV-bpA) under the RSV-LTR promoter control at the Not I and EcoR V sites. Recombinant adenovirus was generated by cotransfection with this plasmid with pJM17 into 293 cells. The positive plaques were purified and further characterized by FACSCAN analysis. Expression of 4-1BB ligand on the Adv.RSV-4-1BB-ligand transduced plasmacytoma cells was highly positive (71% and mean 15) and there was no significant increase in the control vector transduced cells.

Detection of IFN γ concentration in the serum

The mice blood was collected by cutting the tail tips of the treated animals at various time points. Serum was then separated by centrifugation. The IFN γ concentration in the mouse serum was detected by ELISA (R&D Inc.).

***In Vitro* cytotoxic assay**

Freshly isolated effector cells were analyzed by both CTL and NK cytolytic assays. While CTL assay required an additional stimulation of the effector cells ($6x10^6$) with irradiated parental tumor ($5x10^5$ cells, receiving 15,000 rads) and recombinant mIL-2 (20 units/ml for 5 days, the NK cytolytic assay directly used freshly isolated MNC to coincubate with ^{51}Cr labeled target cells ($150\mu Ci/5x10^6$ cells) for 4 hours at 37°C at various effector to target cell ratios. After incubation, the radioactivity released in the supernatant was measured in a gamma counter. The percentage of cell lysis was calculated as: (experimental release-spontaneous release)/(maximal release-spontaneous release) x 100. The standard deviation for the triplicate wells is less than 7%.

***In Vitro* cell depletion and blocking**

In vitro depletion of T cell and NK was accomplished by using Thy1.2 hybridoma supernatant (ATCC) and purified DX5 or anti-asialo GM1 antibody (Pharmigen and Wako Co., respectively). The effector cells were incubated with proper concentration of antibodies on ice for 45 minutes and depleted with rabbit complement (Pel-Freez) for two 30 minutes cycles at 37°C. The complement to the effector cells alone did not affect target cell lysis. Optimal concentration of antibodies and complement were used and verified by flow cytometry. There were less than 1% CD3 positive cells present after Thy1.2 T cell depletion. The efficacy of the NK depletion procedure was confirmed by a direct cytolytic assay against YAC-1 using splenocytes from Poly I:C treated animals. In vitro blocking of CD3+ effector population was accomplished by using purified 145-2C11 (Pharmingen). The cells were blocked with 2 μg/1 x 10^6 cells for 45 minutes prior to incubating with the target cells.

Macroscopic metastatic tumor model

In order to evaluate the systemic anti-tumor effect of the new combination therapy, a 9 day pre-existing macroscopic metastases model was established. Briefly, $3x10^4$ MCA26 cells were injected through the tail veins one day prior to the usual liver tumor implantation. After eight days, the animals were divided into two groups, one to receive the combination therapy and the other to receive no treatment. On the day of virus injection, several mice were sacrificed for biopsy and pathological observation. 100-200 tumor modules could be observed on the lung surfaces, with sizes ranging from 0.5-0.8mm in diameter. There were also many nodules present on the walls of gastrointestinal tract and lymph node.

6.2 RESULTS

Anti-4-1BB antibodies significantly enhance the anti-tumor effect of IL-12 gene therapy

Intratumorally administered Adv.mIL-12 was found to significantly prolong the median survival time of tumor bearing animals, with 25% of the animal becoming tumor free after a single treatment. In an attempt to improve this long-term anti-tumor effect mediated by IL-12, Adv.mIL-12 gene therapy was combined with an agonistic anti-4-1BB antibody administered intraperitoneally. After 120 days, the long-term survival of mice intrahepatically implanted with $7x10^4$ MCA tumor cells and treated with ADV.mIL-12 + anti-4-1BB antibody, ADVmIL-12 + control antibody, control vector (DL312) + anti-4-1BB antibody, or control vector (DL312) alone was determined. 80-100% of mice in receiving the combination of ADV.mIL-12 and anti-4-1BB antibody remained alive, at Adv.mIL-12 doses ranging from $0.2x10^8$ to $3.6x10^8$ ADV.mIL-12 pfu/mouse (Figure 1). Only 42.8% of the animals treated with 3.6x x 10^8pfu of Adv.mIL-12+control Ig survived as compared to 100% survival at this dose of Adv.mIL-12+anti-4-1BB, and only 14.5% of control vector (DL312)+anti-4-1BB treated animals survived. Thus, the therapeutic effect of combination therapy (0.2 x 10^8 - 3.6x 10^8 pfu of Adv.mIL-12+anti4-1BB) is significantly better than either treatment alone ($p<0.0001$).

Further, the long-term survival of BALB/c mice bearing JC breast carcinoma liver metastases treated 87, 60, and 22% of tumor bearing mice treated with IL-12 + anti-4-1BB, DL312 control vector + anti-4-1BB, or IL-12 + rat Ig was evaluated (Figure 2). 87%, 60%, and 22% of tumor bearing mice treated with IL-12 + anti-4-1BB, DL312 control vector + anti-4-1BB, or IL-12 + rat Ig, respectively, showed long-term survival ($P=0.02$ (IL-12 + anti-4-1BB versus DL312 + anti-4-1BB; $P<0.0001$ (IL-12 + anti-4-1BB versus IL-12 + rat Ig); $P=0.129$ (DL312 + anti-4-1BB versus IL-12 + rat Ig); logrank test). All mice in the control group died within 60 to 70 days after tumor cell inoculation ($P<0.0001$ comparing all groups with DL312 + rat Ig; logrank test). Thus, the therapeutic effect of IL-12 plus anti-4-1BB

antibody results in a better method of treating tumors than IL-12 or anti-4-1BB antibody alone.

4-1BB Ligand Can Mimic the Agonistic Antibody and Achieve the Synergistic Effect with Adv.mIL-12 Mediated Gene Therapy

To determine if whether the anti-4-1BB antibody can be replaced with a recombinant adenoviral vector expressing 4-1BB ligand, recombinant adenoviral vectors expressing 4-1BB ligand and Adv.mIL-12 were co-delivered at the tumor site. The recombinant adenoviral vector expressing 4-1BB ligand was injected into pre-established hepatic MCA 26 tumors at $1x10^9$ or $5x10^8$ pfu/animal alone and/or a sub-optimal dose of the Adv.mIL-12 vector at $2x10^8$ pfu/mouse. All animals treated with the control vector and 4-1BB ligand died within 32 days (Figure 3). The medium survival rate for Adv.mIL-12 was only 28 days. However, the combined application of Adv.4-1BB ligand and Adv.mIL-12 resulted in longer survival than either treatment alone ($p<0.042$). The results indicate that 4-1BB ligand and mIL-12 vectors have together generate an effective anti-tumor immunity in mice with pre-established hepatic MCA 26 tumors.

To determine whether the effect of 4-1BB ligand treatment in combination with mIL-12 treatment results in long-term survival in mice having other types of tumors, mice having pre-established JC breast carcinoma liver metastases were analyzed for long-term survival (Figure 4). 78%, 22%, and 13% of animals receiving IL-12-12 + 4-1BBL, IL-12 + DL312, and 4-1BBL + DL312, respectively, were long-term survivors (P=0.016 (IL-12 + 4-1BBL versus IL-12 + DL312); P=0.004 (IL-12 + 4-1BBL versus 4-1BBL + DL312); P=0.515 (IL-12 versus 4-1BBL); logrank test). All animals in the control group died within 80 to 90 days (P=0.0002 (IL-12 + 4-1BBL); P=0.011 (IL-12); P=0.132 (4-1BBL); logrank test). These results confirm that the combination of 4-1BB ligand and mIL-12 vectors together result in a more effective anti-tumor immunity than either 4-1BB ligand or mIL-12 alone.

Challenge experiments with parental tumor cells

The persistence of systemic anti-tumor immunity was tested in long-term (>120 days) surviving animals after ADV/IL-12 + ADV/4-1BBL, ADV/IL-12 + anti-4-1BB, ADV/IL-12 or anti-4-1BB alone treatment. JC parental tumor cells ($1x10^5$) and MCA26 cells ($7x10^4$) were implanted subcutaneously (s.c.) on left and right flanks, respectively. Animals were observed for tumor formation and rate of tumor growth. "Naive" BALB/c mice that have never been exposed to JC or MCA26 cells were used to assess the normal growth of a s.c. JC or MCA26 tumor. All naive animals grew s.c. JC or MCA26 tumors. 29% of IL-12 + 4-1BBL, 50% of IL-12 + DL312 or anti-4-1BB + D1 312, and 63% of IL-12 + anti-4-1BB treated animals formed a JC tumor (Figure 5). Compared to naive animals, only the results of IL-12 + 4-1BBL group are significant (P=0.007, Fischer's exact test). However, the rate of

JC tumor growth in each long-term surviving animal was dramatically decreased in comparison to naive controls.

Rejection of Macroscopic Lung Metastases of Colon Carcinoma After Combination Treatment in Animals with Hepatic Tumors

Rejection of macroscopic lung metastases of colon carcinoma after combination therapy, an animal model with pre-established multiple macroscopic tumor nodules in the lung that range from 0.5 to 0.8 mm in diameter was subjected to the test. Animals receiving tail vein infusion of $3x10^4$ MCA26 cells developed multiple lesions in the lung, and 100% of them dies within 32 days. However, all the liver and lung tumor bearing animals receiving the combination treatment in the liver tumor survived well after 70 days (Figure 6). The results strongly suggest that systemic anti-tumor immunity generated from the combination therapy was capable of eradicating pre-existing metastatic tumor in distant organs. ($p<0.0011$) by logrank test.

Anti-4-1BB antibodies and Adv.mIL-12 synergistically activate anti-tumor natural killer cells

To define the synergistic action between cytokines and activation molecules, a kinetic study of direct cytolytic assay from various animal treatment group was performed (Figure 7A).

Mononuclear cells (MNC) were isolated from the liver of the treated mice at various time points (day 0, 2, 4, 7 and 14) after gene delivery and directly assayed for their cytolytic activity against the parental MCA26 tumor cells. Adv.mIL-12 or anti-4-1BB treated animals resulted in little cytolytic activity against the parental tumor cells, which is significantly elevated in the animals after combination treatment. To identify the responsible immune cell type, the assay was repeated using MNC from animals that received the combined treatment at day 2, but after depletion of NK (DX5), or T-cell (Thy1.2), or CD4+ (GK1.5) T-cell. Depletion with NK completely abolished cytolytic response, while depletion of total $CD8^+$ cells but not CD4+ T-cells reduced some of the cytolytic activity (Figure 7B). The results indicate that NK cells and maybe some T-cells are involved in this synergistic tumor killing.

The long-term maintenance of anti-tumor activity requires both NK and T-cell

To determine which cells were responsible for the maintenance of anti-tumor activity and long-term survival of the animals after combination treatment, *in vivo* lymphocyte subset depletion was performed in the surviving animals prior to challenge with parental tumor cells administered at a distant site. Tumorgenic doses of parental MCA26 tumor ($7x10^4$ cells) were implanted subcutaneously on the flanks of the long-term survivors and naive mice as control. The animals were observed for tumor formation over a four-week period, and the results were compared to the control Ig treated group by Fisher Exact test (Figure 8). The

challenge results showed that 100% (8/8) of the naive animals formed subcutaneous tumor, and only 14.2% (1/7) of the control Ig treated group formed tumor, suggesting that long-term anti-tumor immunity is maintained in most animals after combination treatment. In the NK or CD8+ depleted groups, 87.5% (7/8) and 100% (8/8) of the animals formed subcutaneous tumors, respectively. The results provided strong evidence that NK ($p<0.0106$) and CD8+ ($p<0.0005$) cells are maintained in the surviving animals, and both are essential in preventing the animals from tumor relapse.

6.3 DISCUSSION

By using the combination therapy in liver tumor models and the macroscopic lung metastases tumor models, applicants have demonstrated that the long-term remission of both hepatic, breast and lung metastatic tumors with hepatic tumor gene therapy treatment. The results described herein provide a new treatment modality for cancer patients especially for those with both hepatic and multiple metastatic tumors in the other organs.

NK cells have been demonstrated to be the major and essential effector of the early anti-tumor response, and both NK and T-cells are required for the long-term tumor eradication. However, only 25% long-term survival was achieved with Adv.mIL-12 alone because only a small percentage of animals would develop long-term CTL response. To improve the long term effect of IL-12 mediated anti-tumor response, combination of Adv.mIL-12 gene therapy with an agonistic antibody to 4-1BB. The 4-1BB signals preferentially induce activated T cell proliferation and lead to the amplification of cytotoxic T cell response (Schuford et al, 1997, *J. Exp. Med.* 186(1): 47-55). Applicants are the first to report the synergistic effects between 4-1BB ligand or anti-4-1BB antibody and IL-12, and bridge the early NK anti-tumor response with long-term CTL developments to achieve better therapeutic effect on both hepatic and metastatic tumors. Moreover, the combination therapy requires less IL-12, at least 10 fold less than the effective dose of IL-12 alone.

The mechanism of 4-1BB on IL-12 activated NK cells is not clearly understood. So far, *in vitro* cell depletion assays have indicated that NK cells and may be some T-cells are involved in the combination treatment. The 4-1BB activated cell population that contributed to development of T helper cell and CTL development still need to be identified.

The present invention is not to be limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All publications cited herein are incorporated by reference in their entirety.

WHAT IS CLAIMED:

1. A pharmaceutical composition for augmenting activated immune cells, comprising a compound that activates one or more cytokine receptors and a compound that activates one or more co-stimulatory molecules expressed on activated immune cells.

2. The pharmaceutical composition of Claim 1 wherein the composition is for the treatment, prevention or inhibition of cancer.

3. The pharmaceutical composition of Claim 1 wherein the composition is for the treatment, prevention or inhibition of an infectious disease.

4. The pharmaceutical composition of Claim 2 wherein at least one of the cytokine receptors is the IL-12 receptor.

5. The pharmaceutical composition of Claim 3 wherein at least one of the cytokine receptors is the IL-12 receptor.

6. The pharmaceutical composition of Claim 2 wherein at least one of the co-stimulatory molecules is 4-1BB.

7. The pharmaceutical composition of Claim 3 wherein at least one of the co-stimulatory molecules is 4-1BB.

8. The pharmaceutical composition of Claim 4 wherein at least one of the co-stimulatory molecules is 4-1BB.

9. The pharmaceutical composition of Claim 5 wherein at least one of the co-stimulatory molecules is 4-1BB.

10. The pharmaceutical composition of Claim 6, 7, 8 or 9 wherein the compound that activates 4-1BB is selected from the group consisting of 4-1BB ligand, nucleic acid molecules encoding 4-1BB ligand, anti-4-1BB antibodies, and nucleic acid molecules encoding molecules anti-4-1BB antibodies.

11. The pharmaceutical composition of Claim 4, 5, 8 or 9 wherein the compound that activates the IL-12 receptor is selected from the group consisting of IL-12, nucleic acid

molecules encoding IL-12, anti-IL-12 receptor antibodies, and nucleic acid molecules encoding anti-IL-12 receptor antibodies.

12. The pharmaceutical composition of Claim 1, 2, 3, 6 or 7 wherein the compound that activates one or more cytokine receptors is selected from the group consisting of IL-12, IL-15, IL-18, anti-IL-12 receptor antibodies, anti-IL-15 receptor antibodies, anti-IL-18 receptor antibodies, nucleic acid molecules encoding IL-12, nucleic acid molecules encoding IL-15, nucleic acid molecules encoding IL-18, nucleic acid molecules encoding anti-IL-12 receptor antibodies, nucleic acid molecules encoding anti-IL-15 receptor antibodies, and nucleic acid molecules encoding anti-IL-18 receptor antibodies.

13. A method of treating or preventing cancer in a subject comprising administering to the subject in which such treatment or prevention is desired a therapeutically effective amount of a compound that activates one or more cytokine receptors and a compound that activates one or more co-stimulatory molecules expressed on activated immune cells.

14. A method of treating or preventing an infectious disease in a subject comprising administering to the subject in which such treatment or prevention is desired a therapeutically effective amount of a compound that activates one or more cytokine receptors and a compound that activates one or more co-stimulatory molecules expressed on activated immune cells.

15. The method of Claim 13 wherein in at least one of the cytokine receptors is the IL-12 receptor.

16. The method of Claim 14 wherein in at least one of the cytokine receptors is the IL-12 receptor.

17. The method of Claim 13 wherein at least one of the co-stimulatory molecules is 4-1BB.

18. The method of Claim 14 wherein at least one of the co-stimulatory molecules is 4-1BB.

19. The method of Claim 15 wherein at least one of the co-stimulatory molecules is 4-1BB.

20. The method of Claim 16 wherein at least one of the co-stimulatory molecules is 4-1BB.

21. The method of Claim 15, 16, 19 or 20 wherein the compound that activates the IL-12 receptor is selected from the group consisting of IL-12, nucleic acid molecules encoding IL-12, anti-IL-12 receptor antibodies, and nucleic acid molecules encoding anti-IL-12 receptor antibodies.

22. The method of Claim 17, 18, 19 or 20 wherein the compound that activates 4-1BB is selected from the group consisting of 4-1BB ligand, nucleic acid molecules encoding 4-1BB ligand, anti-4-1BB antibodies, and nucleic acid molecules encoding anti-4-1BB antibodies.

23. The method according to Claim 16, 17, 19 or 20 wherein the compound that activates the IL-12 receptor is expressed by a recombinant adenovirus.

24. The method according to Claim 17, 18, 19 or 20 wherein the compound that activates 4-1BB is expressed by a recombinant adenovirus.

25. The method according to Claim 13, 14, 15, 16, 17, 18, 19 or 20 in which the subject is human.

ABSTRACT

The present invention the present invention relates to methods and compositions for the treatment, prevention or inhibition of diseases and disorders, including cancer, inflammatory diseases or disorders, and infectious diseases, comprising compounds which augment activated immune cells, *i.e.*, T-cells and natural killer ("NK") cells. In particular, the present invention relates to methods and compositions for the treatment, prevention or inhibition of diseases and disorders, including cancer, inflammatory diseases or disorders, and infectious diseases, comprising the administration of a compound that activates one or more cytokine receptors and a compound that activates of one or more co-stimulatory molecules expressed by activated immune cells, *i.e.*, activated T-cells.

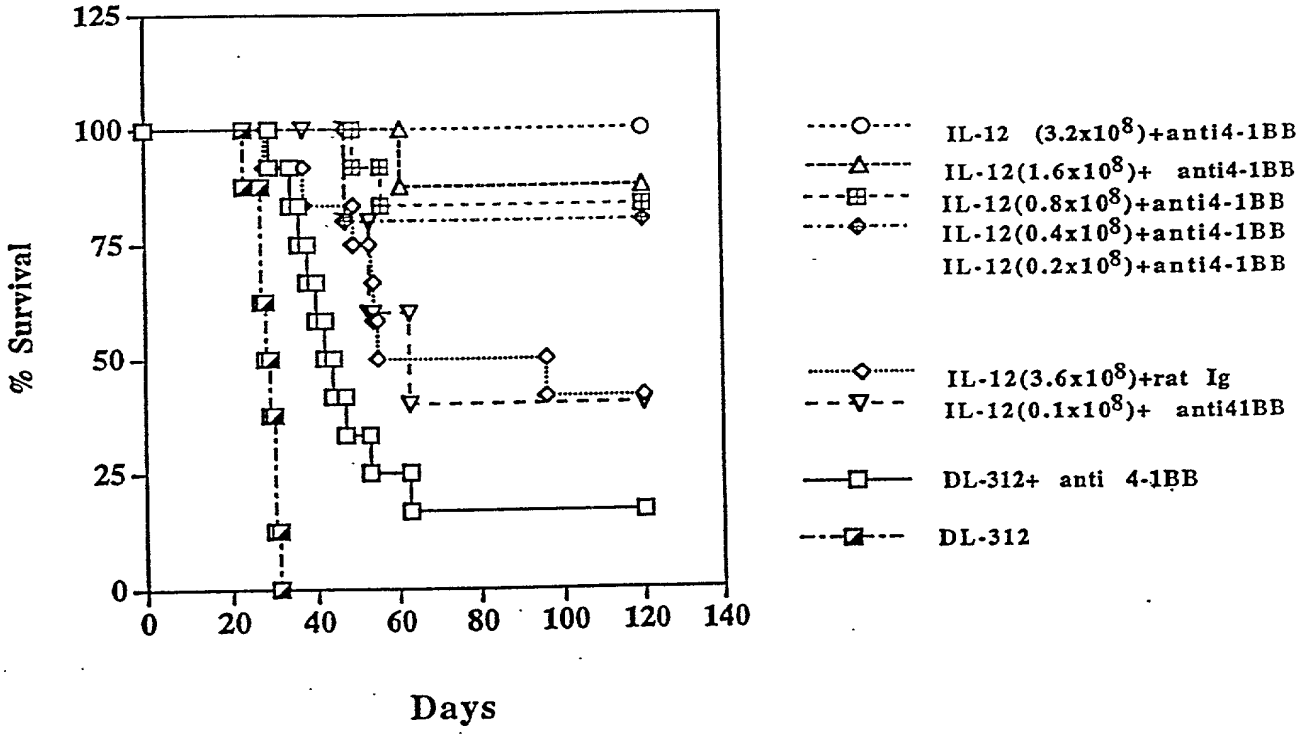
125
100
75
50
25
0
% Survival
0
20
40
60
80
100
120
140
Days
IL-12 (3.2x10^8)+anti4-1BB
IL-12(1.6x10^8)+ anti4-1BB
IL-12(0.8x10^8)+anti4-1BB
IL-12(0.4x10^8)+anti4-1BB
IL-12(0.2x10^8)+anti4-1BB
IL-12(3.6x10^8)+rat Ig
IL-12(0.1x10^8)+ anti41BB
DL-312+ anti 4-1BB
DL-312

FIG. 1

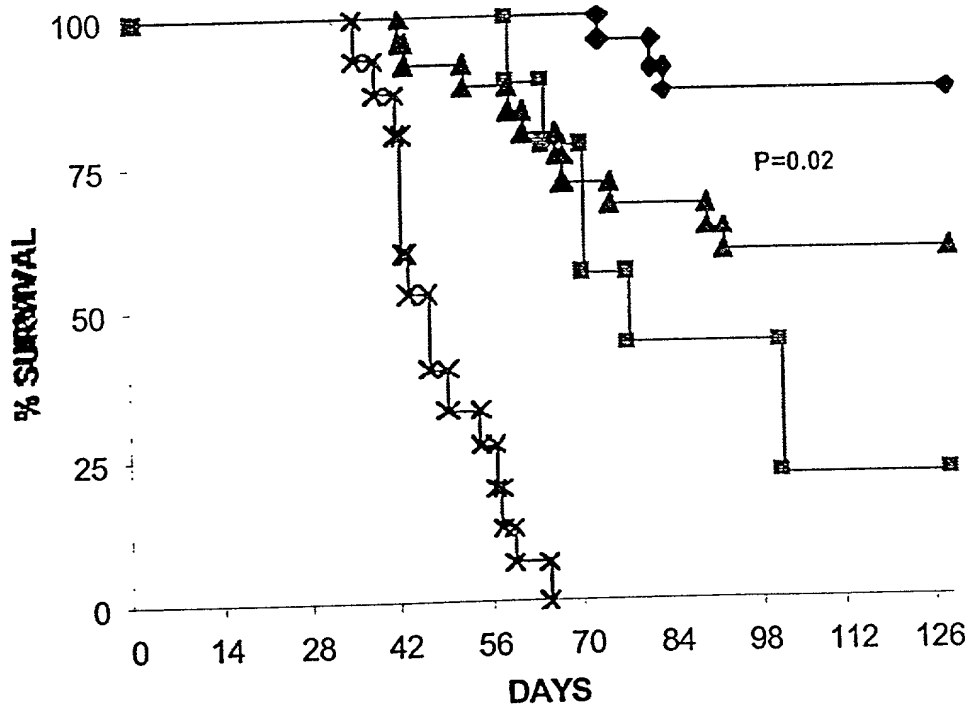

FIG. 2

For 100% alive animals taken animals after 10 days (exluded toxical death).

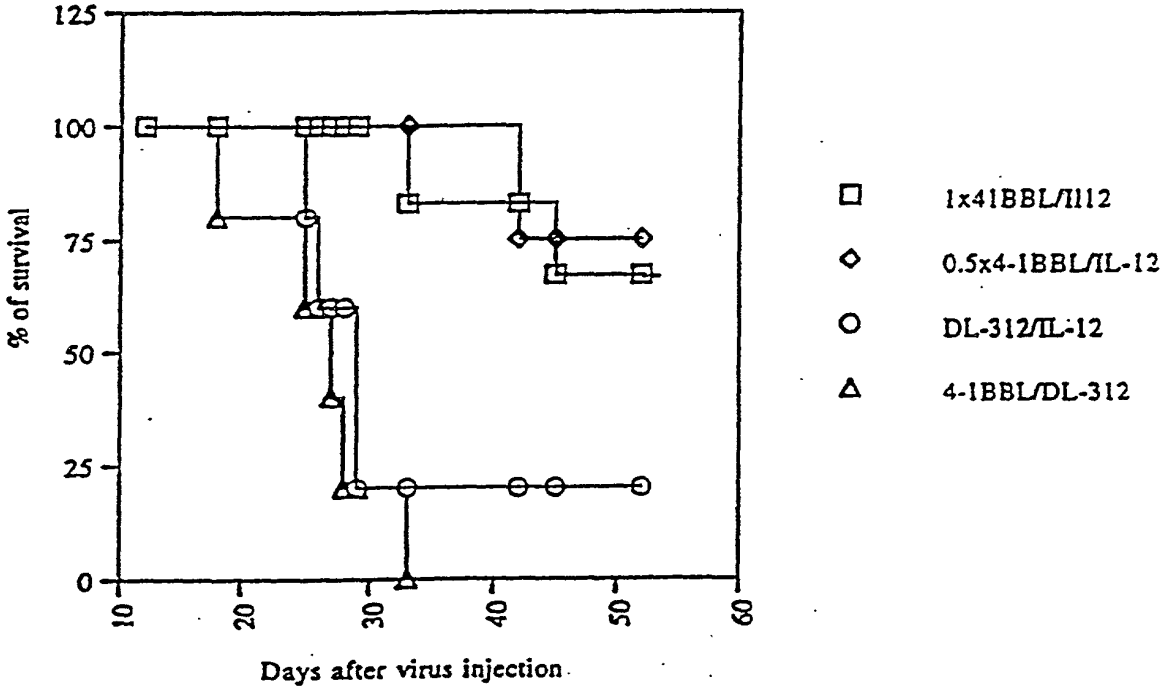

FIG. 3

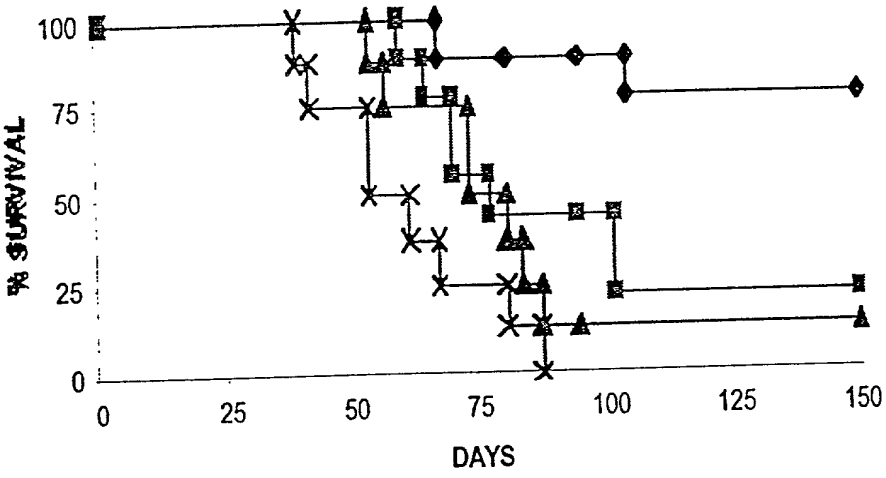

FIG. 4

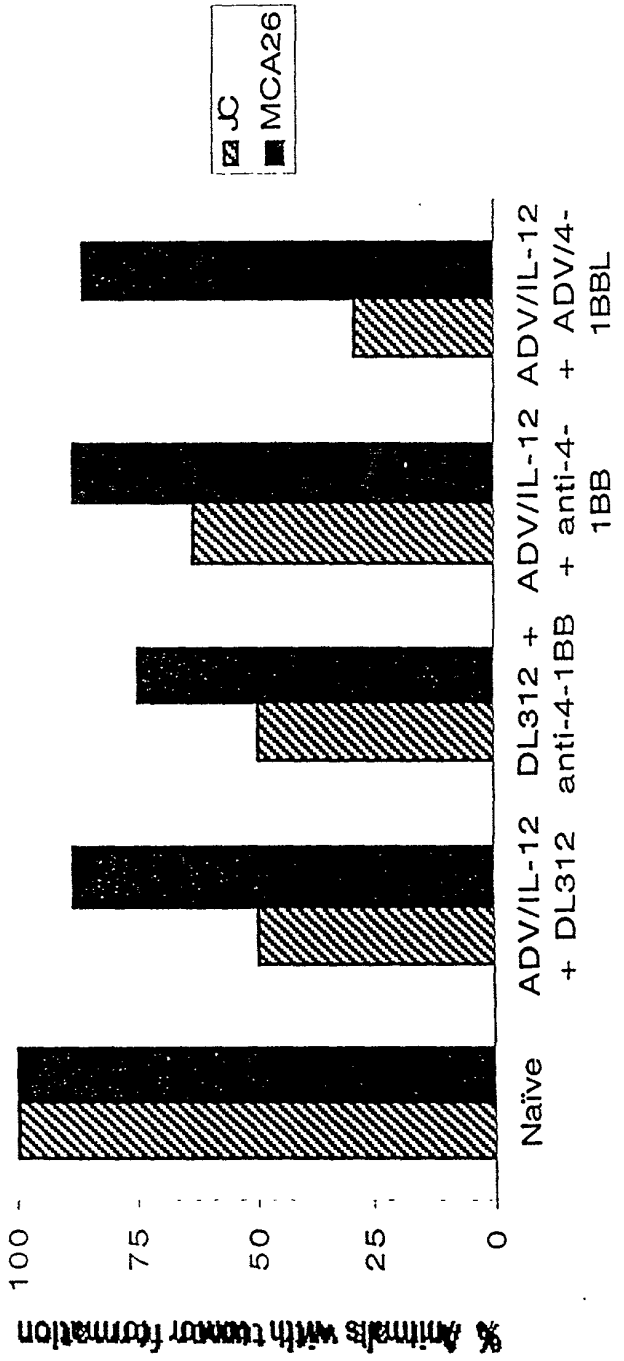
JC
MCA26
% Animals with tumor formation
100
75
50
25
0
Naïve
ADV/IL-12 + DL312
DL312 + anti-4-1BB
ADV/IL-12 + anti-4-1BB
ADV/IL-12 + ADV/4-1BBL

FIG. 5

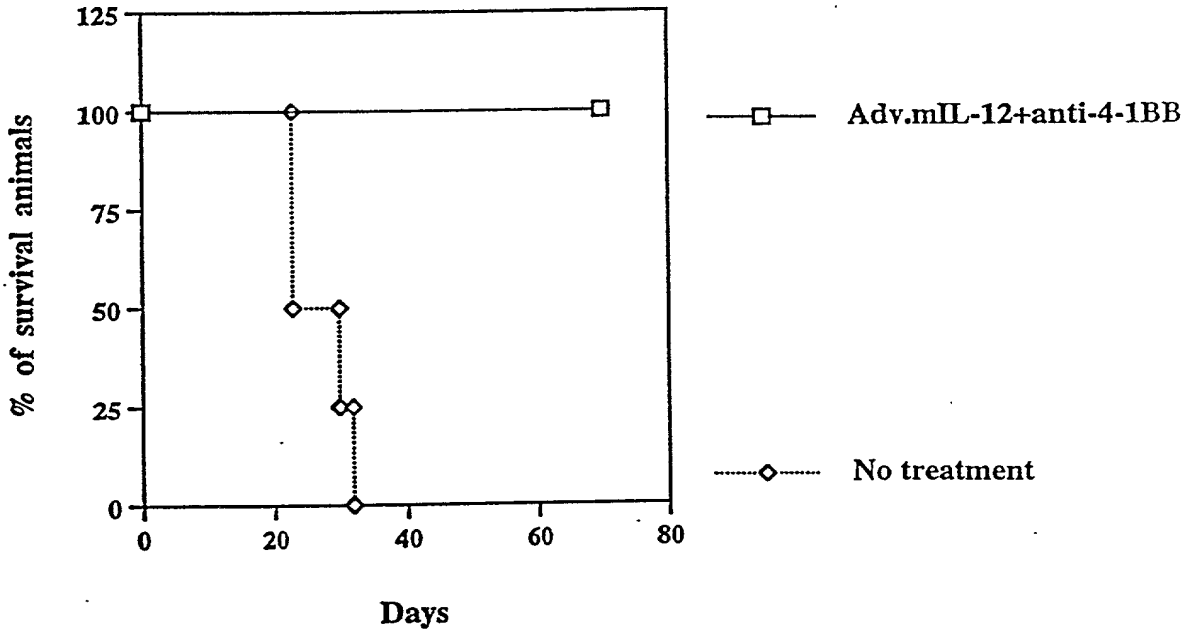
125
100
75
50
25
0
% of survival animals
0
20
40
60
80
Days
Adv.mIL-12+anti-4-1BB
No treatment

FIG. 6

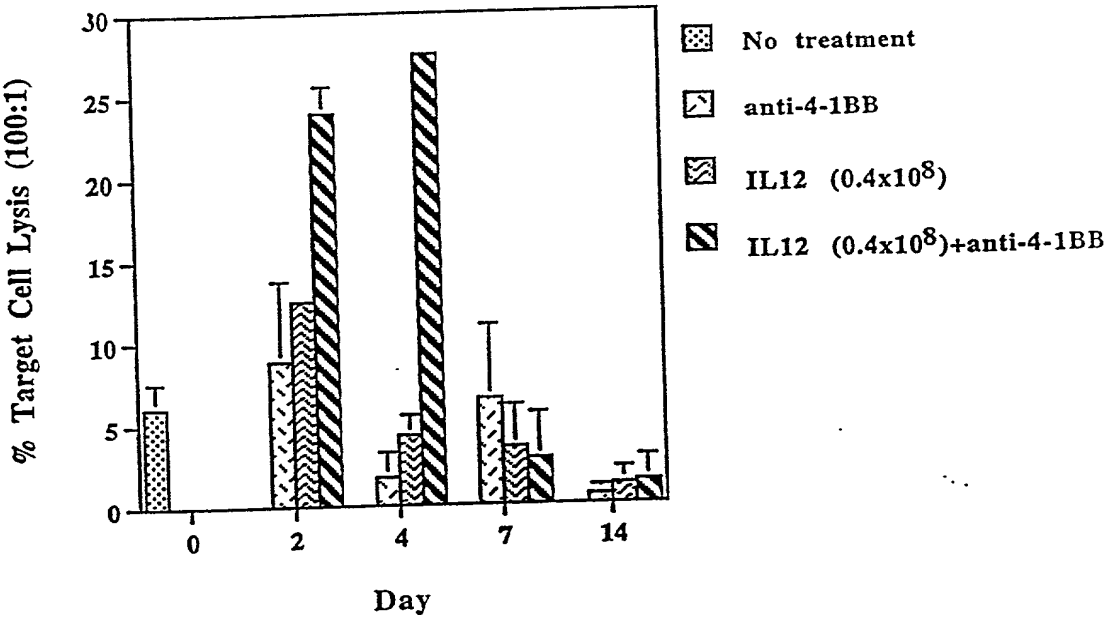
% Target Cell Lysis (100:1)
30
25
20
15
10
5
0
0
2
4
7
14
Day
No treatment
anti-4-1BB
IL12 (0.4x10^8)
IL12 (0.4x10^8)+anti-4-1BB

FIG. 7A

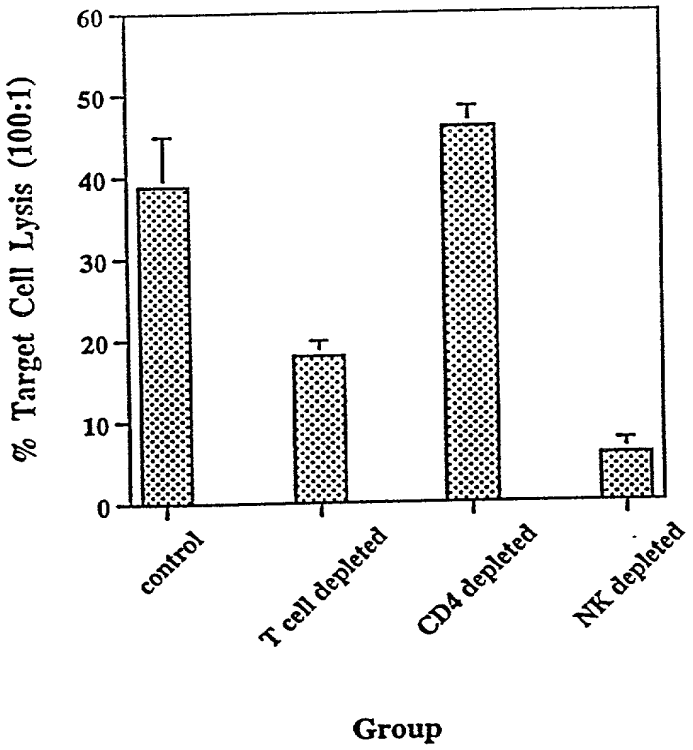
% Target Cell Lysis (100:1)
60
50
40
30
20
10
0
control
T cell depleted
CD4 depleted
NK depleted
Group

FIG. 7B

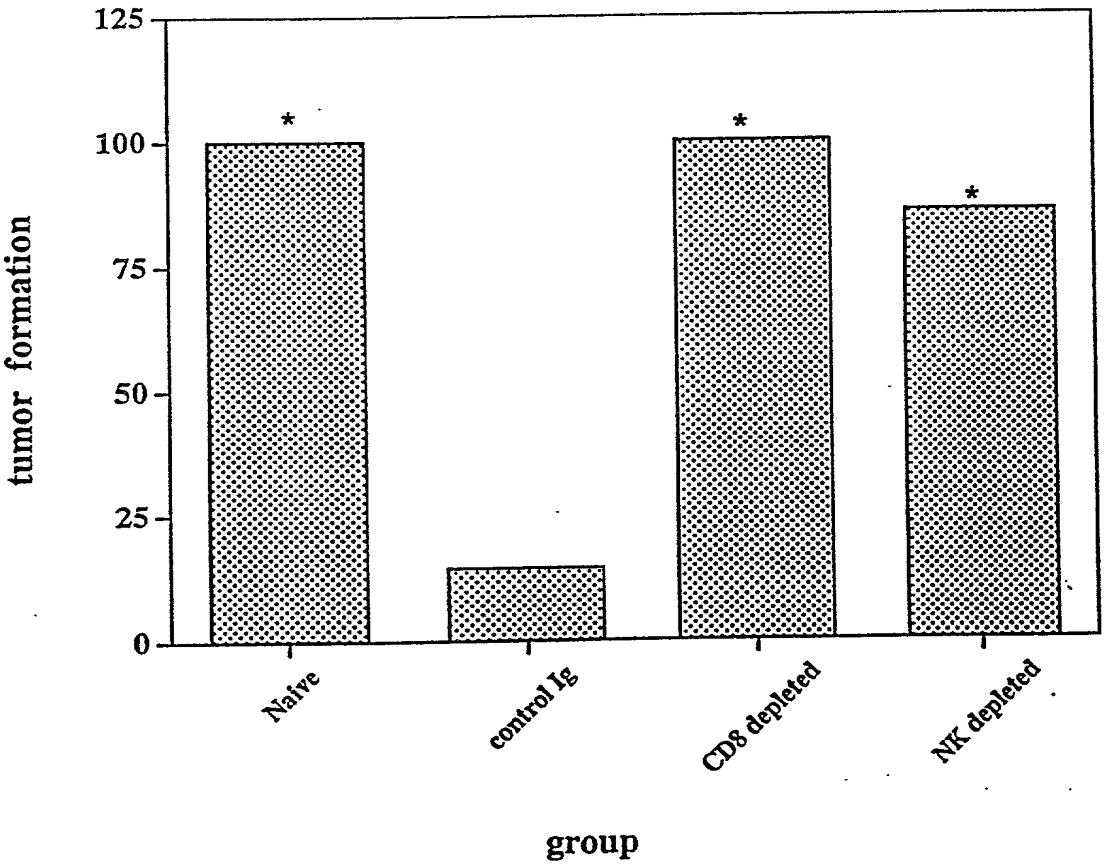
125
100
75
50
25
0
tumor formation
*
*
*
Naive
control Ig
CD8 depleted
NK depleted
group

FIG. 8

DECLARATION
AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below at 201 et seq. underneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

COMBINATION THERAPY OF CANCER BY THE ACTIVATION OF CO-STIMULATORY MOLECULES EXPRESSED BY IMMUNE CELLS AND CYTOKINES

and for which a patent application:
☐ is attached hereto and includes amendment(s) filed on *(if applicable)*
☐ was filed in the United States on as Application No *(for declaration not accompanying application)*
with amendment(s) filed on *(if applicable)*
☐ was filed as PCT international Application No on and was amended under PCT Article 19 on *(if applicable)*

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
			YES ☐ NO ☐
			YES ☐ NO ☐

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

APPLICATION NUMBER	FILING DATE
60/115,992	January 15, 1999

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED

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204	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME	
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	POST OFFICE ADDRESS	STREET	CITY	STATE OR COUNTRY	ZIP CODE
205	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME	
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	POST OFFICE ADDRESS	STREET	CITY	STATE OR COUNTRY	ZIP CODE
206	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME	
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	POST OFFICE ADDRESS	STREET	CITY	STATE OR COUNTRY	ZIP CODE

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon

SIGNATURE OF INVENTOR 201 Shu-Hsia Chen	SIGNATURE OF INVENTOR 202 Savio L.C Woo	SIGNATURE OF INVENTOR 203
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SIGNATURE OF INVENTOR 204	SIGNATURE OF INVENTOR 205	SIGNATURE OF INVENTOR 206
DATE	DATE	DATE